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(54) Title: MAST CELL GROWTH FACTOR

(57) Abstract

There is disclosed a mammalian Mast Cell Growth Factor (MGF) polypeptide, derivatives thereof, DNA sequences, recombinant DNA molecules and transformed host cells that produce MGF polypeptides. More particularly, this invention provides isolated mammalian MGF polypeptides and derivatives thereof that regulate early events in stem cell proliferation. Moreover, MGF polypeptides and derivatives thereof bind to a protein expression product of a c-*kit* proto-oncogene and/or stimulate mast cell proliferation.

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GGC TGG ATC GCA GCG CTG CCT TTC CTT ATG AAG AAG ACA CAA ACT   45
                                Met Lys Lys Thr Gln Thr  -20

TGG ATT CTC ACT TGC ATT TAT CTT CAG CTG CTC CTA TTT AAT CCT   90
Trp Ile Leu Thr Cys Ile Tyr Leu Gln Leu Leu Leu Phe Asn Pro   -5

CTC GTC AAA ACT GAA GGG ATC TGC AGG AAT CGT GTG ACT AAT AAT  135
Leu Val Lys Thr Glu Gly Ile Cys Arg Asn Arg Val Thr Asn Asn   11

GTA AAA GAC GTC ACT AAA TTG GTG GCA AAT CTT CCA AAA GAC TAC  180
Val Lys Asp Val Thr Lys Leu Val Ala Asn Leu Pro Lys Asp Tyr   26

ATG ATA ACC CTC AAA TAT GTC CCC GGG ATG GAT GTT TTG CCA AGT  225
Met Ile Thr Leu Lys Tyr Val Pro Gly Met Asp Val Leu Pro Ser   41

CAT TGT TGG ATA AGC GAG ATG GTA GTA CAA TTG TCA GAC AGC TTG  270
His Cys Trp Ile Ser Glu Met Val Val Gln Leu Ser Asp Ser Leu   56

ACT GAT CTT CTG GAC AAG TTT TCA AAT ATT TCT GAA GGC TTG AGT  315
Thr Asp Leu Leu Asp Lys Phe Ser Asn Ile Ser Glu Gly Leu Ser   71

AAT TAT TCC ATC ATA GAC AAA CTT GTG AAT ATA GTG GAT GAC CTT  360
Asn Tyr Ser Ile Ile Asp Lys Leu Val Asn Ile Val Asp Asp Leu   86

GTG GAG TGC GTG AAA GAA AAC TCA TCT AAG GAT CTA AAA AAA TCA  405
Val Glu Cys Val Lys Glu Asn Ser Ser Lys Asp Leu Lys Lys Ser  101

TTC AAG AGC CCA GAA CCC AGG CTC TTT ACT CCT GAA GAA TTC TTT  450
Phe Lys Ser Pro Glu Pro Arg Leu Phe Thr Pro Glu Glu Phe Phe  116

AGA ATT TTT AAT AGA TCC ATT GAT GCC TTC AAG GAC TTT GTA GTG  495
Arg Ile Phe Asn Arg Ser Ile Asp Ala Phe Lys Asp Phe Val Val  131

GCA TCT GAA ACT AGT GAT TGT GTG GTT TCT TCA ACA TTA AGT CCT  540
Ala Ser Glu Thr Ser Asp Cys Val Val Ser Ser Thr Leu Ser Pro  146

GAG AAA GGG AAG GCC AAA AAT CCC CCT GGA GAC TCC AGC CTA CAC  585
Glu Lys Gly Lys Ala Lys Asn Pro Pro Gly Asp Ser Ser Leu His  161

TGG GCA GCC ATG GCA TTG CCA GCA TTG TTT TCT CTT ATA ATT GGC  630
Trp Ala Ala Met Ala Leu Pro Ala Leu Phe Ser Leu Ile Ile Gly  176

TTT GCT TTT GGA GCC TTA TAC TGG AAG AAG AGA CAG CCA AGT CTT  675
Phe Ala Phe Gly Ala Leu Tyr Trp Lys Lys Arg Gln Pro Ser Leu  191

ACA AGG GCA GTT GAA AAT ATA CAA ATT AAT GAA GAG GAT AAT GAG  720
Thr Arg Ala Val Glu Asn Ile Gln Ile Asn Glu Glu Asp Asn Glu  206

ATA AGT ATG TTG CAA GAG AAA GAG AGA GAG TTT CAA GAA GTG TAA  765
Ile Ser Met Leu Gln Glu Lys Glu Arg Glu Phe Gln Glu Val      220

TTG TGG CTT GTA TCA                                         780
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TITLE

MAST CELL GROWTH FACTOR

TECHNICAL FIELD OF THE INVENTION

This invention relates to mammalian Mast Cell Growth Factor polypeptides ("MGF"), nucleotide sequences encoding MGF and certain derivatives, analogs and allelic variants thereof, processes for production of MGF polypeptides by recombinant DNA technology or by purification from culture media of cell lines which constitutively produce MGF, and pharmaceutical compositions comprising MGF and an additional growth factor. More specifically, the invention provides isolated mammalian nucleotide sequences encoding MGF and derivatives thereof that regulate early events in stem cell proliferation. More specifically, the present invention discloses murine and human nucleotide sequences encoding murine and human MGF, and also nucleotide sequences capable of hybridizing to disclosed and/or deposited nucleotide sequences and encoding MGF and derivatives or analogs thereof having MGF biological activity.

BACKGROUND OF THE INVENTION

Hematopoietic growth factors regulate the growth and maturation of various lineages of blood cells. All known blood cells are believed to develop from a single class of precursor cells called stem cells. Each hematopoietin causes specific classes of blood cells to differentiate and proliferate. When a stem cell divides in the bone marrow, it can replicate itself as a stem cell or become committed to a particular developmental pathway. As a result of commitment, a stem cell displayss receptors on its cell surface that enables it to respond to certain hormonal signals. Such signals push the cell further down a pathway leading to terminal differentiation. As differentiation proceeds, the first recognizable precursors appear, including erythroblasts (red cell precursors) and myeloblasts (precursors of granulocytes and monocytes).

Scientific understanding of hematopoiesis has been aided by developments in molecular biology. A number of glycoproteins have been identified which regulate cell development at various levels within the hematopoietic stem and progenitor cell hierarchy. The majority of growth factors that have been identified influence relatively late stages of differentiation and regulate the number and function of mature differentiated hematopoietic elements. Identification of the factors controlling more primitive elements of the hierarchy has proven difficult, due to the very low frequency of these progenitor cells, and a lack of phenotypic markers restricted to stem cells.

By definition, a "true" pluripotent hematopoietic stem cell (PHSC) is a cell capable of extensive proliferation, self-renewal, and the ability to mediate long-term reconstitution of all hematopoietic lineages. PHSC are extremely rare cells in the hematopoietic system, which are

not actively cycling under normal circumstances. In the mouse, attempts to purify PHSC have centered on the purification of spleen colony-forming cells (CFU-S) (Spangrude et al., *Science 241*:58 (1988)). However, others have demonstrated that CFU-S cells are not the "true" PHSC as assessed by their capacity for long-term reconstitution. Other studies have suggested a cell more primitive than a CFU-S which then gives rise to daughter CFU-S cells. Such a cell has been referred to as a "pre-CFU-S." Other cells have certain stem cell-like features, including high proliferative potential colony-forming cells (HPP-CFC), a mixed colony-forming cell (CFU-GEMM), a blast cell CFC, and long-term culture initiating cells. These later cell types fail to fulfill all of the criteria for a "true" stem cell, but point to the complexity and heterogenous functional capability of primitive hematopoietic cells.

Progenitor cells are the immediate progeny of stem cells, but differ in two respects. First, progenitor cells have a restricted capacity for self-renewal, and secondly, progenitor cells are committed irreversibly to a single lineage of hematopoietic differentiation (or two, in the case of many granulocyte-macrophage progenitor cells). Progenitor cells are the cells responsible for forming the majority of observable cell colonies in experimental cultures of hematopoietic cells. Examples of progenitor cells include, granulocyte-macrophage progenitors, eosinophil progenitors, megakaryocyte progenitors, multipotential progenitors and erythroid progenitors.

Hematopoiesis *in vivo* is regulated both by feedback signals generated from peripheral tissues, i.e., long-range regulation, and by interaction between stromal cells in the bone marrow and target hematopoietic stem or progenitor cells. Regulation is likely due to the transfer of regulatory molecules from cell to cell. Stromal cell lines produce a variety of known hematopoietic factors, such as interleukin-7 (IL-7), granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte CSF (G-CSF), macrophage CSF (M-CSF), interleukin-1 (IL-1α and IL-1β), interleukin-6 (IL-6) and leukemia inhibitory factor (LIF).

There is a continuing need in the art to identify hematopoietic regulatory factors and the cellular receptors that bind to each regulatory factor. One such receptor is a tyrosine kinase receptor encoded by the c-*kit* proto-oncogene (Yarden et al. *EMBO J 6*:3341 1987). This receptor is structurally related to the receptor for M-CSF, (Qui et al. *EMBO J. 7*:1003 1988) which is encoded by the c-*fms* proto-oncogene (Sherr et al. *Cell 41*:665 1985). Prior to the present invention, the ligand for the c-*kit* receptor had not yet been identified or characterized in the art.

Mice with mutations at the Steel or *W* locus display a similar phenotype characterized by a reduction of pluripotent hematopoietic stem cells (PHSC), anemia, a deficiency of mast cells, and defects in gametogenesis and pigmentation (Russell *Adv. Genet. 20*:357 1979). The hematopoietic defect in *W* mice is intrinsic to the PHSC, whereas that of the Steel mouse is the result of a microenvironmental aberration. Recent studies have shown that the *W* locus on murine chromosome 5 encodes the c-*kit* proto-oncogene (Chabot et al. *Nature 335*:89 1988;

Geissler et al. *Cell 55*: 185 1988). Previous studies have shown that bone marrow from Steel mice transplanted into *W* recipients resulted in a hematologically normal phenotype (Bernstein et al. *Ann. N.Y. Acad. Sci. 149*:475 1968) and has led to the hypothesis that the gene product of the Steel locus is the ligand for c-*kit* (Chabot et al. *Nature 335*:88 1988; Geissler et al. *Cell 55*: 185 1988).

In mice, mutations at the Steel (*Sl*) locus identify a gene essential for the development of neural crest-derived melanocytes, primordial germ cells and hematopoietic stem cells. These cell populations form in *Sl* homozygous embryos, but fail to proliferate or survive, and thus the animals are black-eyed, white, sterile and severely anemic. Several alleles are lethal when homozygous due to failure of erythropoiesis. The lethal period begins at the thirteenth day of gestation after hematopoiesis has commenced in the normal fetal liver. Heterozygote animals are generally viable but differ in severity on three different affected lineages.

Graft experiments have demonstrated that the *Sl* gene acts in the extracellular environment rather than in the affected cells themselves. For example, when *Sl/Sl* premigratory neural crest cells (precursors of melanocytes) are grafted alongside wild type (+/+) skin, they give rise to pigmented melanocytes that populate and pigment skin hair follicles, whereas the converse experiment results in unpigmented hairs. Similarly, hematopoietic stem cells from anemic Sl/Sl^d animals can repopulate stroma and rescue a lethally-irradiated syngeneic host. However, +/+ stem cells fail to rescue a Sl/Sl^d anemia. Although grafting experiments of primordial germ cells have not been possible, studies of Sl/Sl^d <-> +/+ chimeras suggest that Sl/Sl^d germ cells are able to populate the gonad and give rise to functional sperm if the appropriate environment is present.

In mice, *W* or dominant white spotting mutations parallel those of the *Sl* series. The *W* mutation in mice affects the same three (primordial germ cells, hematopoietic stem cells and neural-crest derived melanocytes) stem cell lineages, erythroid cells and mast cells. Due to severe anemia the *W* mutation is generally lethal in the homozygous condition. Grafting experiments, much like those carried out for *Sl* mutations, suggest that the site of action for the *W* gene is within the affected cells themselves rather than within the extracellular environment. Neural crest cells from *W/W* embryos grafted alongside +/+ skin are unable to give rise to melanocytes, whereas +/+ neural crest cells form melanocytes that populate and pigment *W/W* skin hair follicle. Hematopoietic stem cells from severely anemic W/W^V animals fail to rescue lethally-irradiated syngeneic hosts but the W/W^V hematopoietic microenvironment efficiently supports +/+ stem cell proliferation. By inference, the *W* gene is thought to act in the same way in primordial germ cells, although no experimental system exists for testing this hypothesis.

Therefore, there is a need in the art to identify and characterize mammalian equivalents and homologies of the gene product of the mouse Steel locus, herein designated MGF. Such potential hematopoietic stromal cell factors are likely to be useful regulatory molecules for

controlling the early growth and differentiation of stem cells. The ligand is likely to be useful for treating aplastic anemia when an individual already has erythropoietin (EPO) available. MGF is likely to be further useful to treat bone marrow suppression or ineffective hematopoiesis. MGF should be useful when used in combination with other factors having colony stimulating biological activity, such as GM-CSF, G-CSF, IL-3, CSF-1, IL-6 and IL-1.

SUMMARY OF THE INVENTION

We have purified, sequenced and cloned cDNAs encoding several forms of a murine and human (i.e., mammalian) MGF, a novel hematopoietin which stimulates a select group of IL-3 dependent mast cell lines and hematopoietic progenitor cells. The nucleotide sequence and deduced amino acid sequence of murine MGF ("mMGF") are disclosed in Figures 1 and 2. (Sequence ID Nos. 1 and 2). The nucleotide sequence and corresponding amino acid sequence of the intracellular region, transmembrane region and the extracellular region of human MGF ("hMGF") are disclosed in Figures 3 and 4 (Sequence I.D. Nos. 3 and 4). Mature hMGF polypeptide begins at a Glu residue 1. The mature extracellular region of hMGF is 156 amino acids in length as shown in Figure 3, terminating at the Asp residue at amino acid 157. There is another form of hMGF resulting from an alternative mRNA splicing event comprising an exon encoding an additional 28 amino acids beginning at amino acid 148. This form is illustrated in Figure 4. The alternative form is provided by clone hMGF-2.4 and has 185 amino acids in its full length mature extracellular region.

The present invention further comprises other mammalian MGF polypeptides having MGF biological activity and encoded by nucleotide sequences which hybridize, under conditions of moderate stringency, to a probe defined by clone 10 (deposited with the ATCC on September 11, 1990 under accession number 68396) and shown in Figure 2, or by mMGF-94 (shown in Figure 1), or which hybridize to human clone MGF-2D (Figure 3) or hMGF-2.4 (Figure 4) under conditions of high stringency or to various human sequence probes described herein. Moreover, the present invention comprises hMGF nucleotide sequences and polypeptides, wherein said nucleotide sequences hybridize, under high stringency conditions, to a probe defined by clone MGF-2D (whose sequence is shown in Figure 3) or to the nucleotide sequence encoding the extracellular portion of the human sequence and shown in Figure 3.

Proliferative responses to mMGF correlated with expression of c-*kit* mRNA. Moreover, purified, radiolabeled mammalian MGF can be cross-linked to responder cells and subsequently immunoprecipitated with an antisera recognizing the carboxy-terminus of the c-*kit* gene product. Therefore, MGF is the ligand for the protein receptor expression product of c-*kit*. Moreover, binding to the polypeptide receptor encoded by the c-*kit* proto-oncogene is a measure of MGF biological activity. The c-*kit* sequences (mouse and human) have been

published (see Background section) and expressed in various cell types as disclosed herein in the Examples.

MGF has a transmembrane region, an extracellular region and an intracellular region. Human MGF has a 27 amino acid transmembrane region beginning with a Ser residue at amino acid 158 (Figure 3, clone MGF-2D) or amino acid 186 (Figure 4, clone MGF-2.4). A transmembrane region typically comprises hydrophobic amino acid residues flanked by charged amino acids. The intracellular region of hMGF begins with a Lys residue at amino acid 185 (Figure 3, clone MGF-2D) or at amino acid 213 (Figure 4, clone MGF-2.4) and is 36 amino acids in length. Another difference between hMGF-2.4 and hMGF-2D is that amino acid 149 of hMGF-2D is a Gly whereas corresponding amino acid (just after the spliced exon sequence) 177 of hMGF-2.4 is an Arg. This amino acid difference is a result of the spliced exon sequence being inserted within a codon. Figures 1, 2, 3 and 4 illustrate nucleotide and amino acid sequences of wild type mMGF (Figures 1 and 2) and hMGF (Figures 3 and 4). Clone hMGF-2D comprises the full coding region of wild type hMGF without the additional 28 amino acid alternative exon region. Clone hMGF-2.4 comprises the full hMGF coding region with the alternative exon sequence. The polypeptide expressed by the extracellular region of clone hMGF-2.4 is called Δ28 MGF.

Mammalian or human hematopoiesis, including megakaryocytopoiesis, are influenced by administration of MGF. However, optimal hematopoiesis is achieved by combination therapy with MGF and other cytokines, including but not limited to GM-CSF, IL-3, a fusion protein comprising both GM-CSF and IL-3, EPO, IL-1α, IL-1β, G-CSF, LIF, IL-6, and IL-7. More specifically, we found that MGF has the ability to augment the action of other cytokines for megakaryocytopoiesis, an activity shared by IL-3 and IL-6, and provides maximal hematopoietic progenitor proliferative stimulus when employed in combination with GM-CSF, IL-3 or GM-CSF/IL-3 fusion proteins.

MGF was found to influence early lymphoid development, early myeloid development, and promote proliferation and sustain hematopoiesis of early stem cells or progenitor cells of the more primitive types.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a cDNA sequence and corresponding amino acid sequence for clone mMGF-94 'Sequence ID No. 1) without the 16 amino acid spliced sequence. Figure 2 shows the nucleotide sequence and corresponding amino acid sequence for murine MGF clone mMGF-10 (Sequence ID No. 2) with the alternative mRNA exon sequence that adds 16 amino acids to the extracellular region of the polypeptide.

Figure 3 illustrates the wild type hMGF cDNA and amino acid sequence as derived from clone hMGF-2D (Sequence ID No. 3) without the 28 amino acid alternative exon region.

This sequence encodes a polypeptide that is also called Δ28 hMGF. Figure 4 illustrates the wild type hMGF cDNA and amino acid sequence as derived from clone hMGF-2.4 (Sequence ID No. 4). The transmembrane region begins with a Ser Ser sequence following an Asp residue and ends with a Leu Tyr Trp sequence just prior to a Lys Lys sequence that begins (N-terminus) the intracellular region. A portion of the 5' non-coding region is also shown. The mature hMGF polypeptide begins with a Glu residue in the extracellular region.

Figure 5 shows a dose-response comparison of BFU-E and CFU-GEMM activities of different concentrations of MGF.

Figure 6a shows the number of cells recovered from purified stem cell fractions after three days in culture with medium alone, mMGF alone, IL-3 alone or IL-3 plus mMGF for early (dull) and late (bright) stem cells. Figure 6b shows the recovery of CFU-S from dull and bright populations of primitive stem cells after 3 days of culture with medium alone, mMGF alone, IL-3 alone or IL-3 plus mMGF.

DETAILED DESCRIPTION OF THE INVENTION

We have discovered a novel mammalian polypeptide that is the ligand for the receptor encoded by the c-*kit* proto-oncogene. More particularly, we have cloned and sequenced a cDNA encoding purified mMGF. We then cloned and sequenced a cDNA corresponding to hMGF. Both MGFs have an extracellular region that contains the c-*kit* binding region, a hydrophobic transmembrane region delineated by charged amino acids on either side and an intracellular region. The present invention further comprises a soluble human MGF polypeptides comprising amino acid sequences corresponding to the extracellular domain or at least a region of the extracellular domain encompassing all four Lys residues.

MGF is a ligand for the gene product of the c-*kit* proto-oncogene. c-*kit* encodes a tyrosine kinase receptor on bone marrow stem cells and other tissues including, for example, primordial germ cells (PGC), melanocytes and developing (embryonic) neural crest cells arising from the neural tube. Thus, MGF activity comprises signaling proliferation and/or differentiation of precursor bone marrow stem cells and a variety of other germ-type cells for developing central and peripheral nervous system, gametogenesis and pigmentation. Moreover, MGF is preferably secreted by bone marrow stromal cells in either precursor or mature forms, or both. There is also a natural soluble form of MGF comprising the extracellular region or a fragment thereof.

Assays for MGF

Isolation and characterization of MGF requires a mammalian cell line that produces MGF, and a receptor cell line for assay that proliferates in response to MGF stimulation. A biological assay for mammalian MGF can use, for example, a growth factor-dependent mast

cell line to look for cell proliferation. Any IL-3 dependent cell line expressing c-*kit* can be used to provide responder cells for a proliferation assay receptor or binding assay for a mammalian MGF. Moreover, fresh mast cells isolated from bone marrow of normal mice express c-*kit* and can similarly be used for receptor binding assays or for proliferation assays for mammalian MGF. In addition, fresh mast cells isolated from marrow samples taken from humans or from mammals can be used to assay for mammalian MGF.

A MGF-dependent cell line can be derived from fresh Thy-1$^+$ marrow cells of NFS/N mice. The NFS/N1 cell line is a mast cell that lacks Thy-1$^+$, ThB, and B220. This cell line responds to purified murine IL-3 and recombinant murine IL-4, but not to natural or recombinant murine GM-CSF, recombinant human G-CSF or murine CSF-1. An IL-3-dependent cell line was obtained from IL-3 responsive cells from an NFS/N mouse strain passaged in a high concentration of WEHI-3 conditioned media ("WEHI-3-CM") containing IL-3 after enrichment of Thy-1$^+$ cells by a fluorescence-activated cell sorter. After passage *in vitro* for several months, the cell line was cloned. Cell line cloning can be accomplished, for example, by limiting dilution and by isolation of individual colonies growing in methylcellulose in response to a combination of WEHI-3-CM and stromal cell-CM. WEHI-3-CM are collected from supernatants of the WEHI-3 murine leukemia cell line (ATCC TIB68) growing in RPMI-1640 medium supplemented with 2% fetal calf serum. The supernatant is dialyzed against distilled water and concentrated five-fold prior to use.

The NFS/N1-MC6 or MC6 cell line provides biological activity assays for murine MGF by proliferating in response to murine MGF as measured by colony formation or by uptake of tritiated thymidine. Similarly, human MGF can be assayed for MGF biological activity by anyone of a number of cell lines that require human IL-3 for growth and express the product of the *c-kit* gene product. Examples of such cells include TF1 cells (Kitamura et al., *J. Cell. Physiol. 140*:323, 1989) and Mo7e cells (Avanzi et al., *Br. J. Haematol. 69*:359, 1988). To conduct a proliferation assay, factor-dependent cells (e.g., MC6 cells, TF1 cells, or mast cells) are placed into replicate microwells in a suitable medium, such as RPMI 1640 supplemented with 10% fetal calf serum. Supernatants from cells secreting putative growth factor are added to the wells and the cells are incubated overnight at 37°C in a 5% CO_2 atmosphere. The cells are next pulsed with 1 μCi of tritiated thymidine and incubated for an additional eight hours. The wells are harvested onto glass fiber filter discs for counting by liquid scintillation. Presence of a growth factor is determined by increased tritium in the cells as an indication of cellular division.

MGF Polypeptides

MGF refers to a family of mammalian polypeptides which are capable of stimulating IL-3 dependent mast cell lines and hematopoietic progenitor cells, and serve as a ligand for the gene product of the c-*kit* proto-oncogene. As used herein, the term MGF includes analogs or

subunits of native mammalian polypeptides with substantially identical or substantially similar amino acid polypeptide sequences which bind to the protein expressed by the c-*kit* proto-oncogene and which induce proliferation of mast cells, for example, the IL-3 dependent murine mast cell line MC6 or human cell line TF1. MGF may consist solely of its extracellular region or a fragment thereof including all four Cys residues of the extracellular domain and may lack a transmembrane region and intracellular domain. The extracellular region of MGF or fragment thereof is a soluble polypeptide. Both human and murine native sequence MGF have been found in two variations, differing by an extra coding region in the extracellular domain of the polypeptide. One human variant (clone MGF 2.4) has an extra 28 amino acids beginning at amino acids 148 in the extracellular domain of the polypeptide. One murine variant (clone mMGF-10) has an extra 16 amino acids beginning with amino acid 148 in its extracellular domain. Similarly, clone hMGF2-D lacks a 28 amino acid sequence in the extracellular domain. The extracellular domain of this polypeptide is called Δ28 hMGF.

Human MGF polypeptides have a full length 185 amino acid extracellular domain. This polypeptide has five glycosylation sites and four Cys residues. The first Cys residue at position 3 of the mature human polypeptide sequence binds to the third Cys residue at position 89 of the human MGF polypeptide sequence. Similarly, the second Cys residue at position 43 binds to the fourth Cys residue at position 138. Δ28 hMGF retains all four Cys residues, but eliminates the fifth glycosylation site. When several amino acids are removed from the C-terminus of hMGF extracellular domain, up to the fourth Cys residue at position 138, biological activity is retained. Similarly, one can remove the first three N-terminal amino acids, up to the first Cys residue at position 3 of mature human MGF, and retain biological activity. Therefore, a human MGF polypeptide having only 136 amino acid residues, but retaining all four Cys residues, retains significant biological activity, comparable to full length 185 amino acid human MGF or Δ28 hMGF. Δ28 hMGF has significant activity.

Murine and human MGF are 87% homologous at the DNA level and 90% homologous at the protein level. However, despite 90% homology at the protein level, there are enough differences in protein structure for human MGF to be 2-3 logs less active in a murine cell (MC6) assay and murine MGF to be 2-4 fold less active in a human (TF1 cell) proliferation assay.

In order to find important amino acid residues involved in receptor binding and conferring species specificity, we made human-murine chimeric MGF polypeptides incorporating various sequence regions of the extracellular domain. We were able to determine that there were at least one and possibly a group of amino acids involved in conferring murine MGF biological activity between *Ssp*I restriction site (between residues 90 and 91) and an *Eco*RI (between residues 138 and 189) restriction site in the human sequence. The protein sequence between these restriction sites contains 50 amino acids, 14 of which are different amino acids between the human and murine MGF polypeptides. We made a murine-human-

murine MGF chimeric protein having murine sequence from the 5' end to the *Ssp* I site, human sequence from the *Ssp* I site to the *Eco*RI site and murine sequence from the *Eco*RI site to the 3' end of the extracellular domain. This chimera was active in a human assay but inactive in a murine assay. The 50 amino acid region was not sufficient (alone) to confer total biological activity but needs to be configured in the context of additional protein.

We investigated 14 different amino acids within the 50 amino acid region. Chimeric constructs were made by a PCR overlap extension method and transfected into COS cells. COS cell supernatants were assayed in murine and human proliferation assays. Based upon these data, we found that the amino acid at position 91 is important to confer species specificity of an MGF polypeptide. Murine MGF has a Glu (no charge) residue at position 91, and human MGF has a Lys (positively charged) residue. Thus, the amino acid residue at position 91 of either human or murine MGF is important for receptor binding or protein conformation (tertiary structure).

One can make analogs of the hMGF molecule which retain MGF biological activity. For example, glycosylation sites can be altered to facilitate expression in yeast or mammalian cell systems. The region surrounding the third Cys at position 89 is important for receptor binding. However, the Val residue at residue 90 can be altered to any other amino acid without affecting biological activity. The adjoining Lys residue at position 91 confers human species specificity to hMGF and requires a Glu residue at this position for murine species specificity.

Human MGF analogs can vary in length from about 135 amino acids to about 185 amino acids constituting the extracellular domain of the hMGF polypeptide. Biologically active hMGF analog polypeptides comprise four Cys residues, but can vary at other positions according to the following sequence:

X_n X_n X_n Cys Arg Asn Arg Val Thr Asn Asn
Val Lys Asp Val Thr Lys Leu Val Ala Asn Leu Pro Lys Asp Tyr
Met Ile Thr Leu Lys Tyr Val Pro Gly Met Asp Val Leu Pro Ser
His Cys Trp Ile Ser Glu Met Val Val Gln Leu Ser Asp Ser Leu
Thr Asp Leu Leu Asp Lys Phe Ser Asn Ile Ser Glu Gly Leu Ser
Asn Tyr Ser Ile Ile Asp Lys Leu Val Asn Ile Val Asp Asp Leu
Val R_1 Cys R_2 Q Glu Asn Ser Ser Lys Asp Leu Lys Lys Ser
Phe Lys Ser Pro Glu Pro Arg Leu Phe Thr Pro Glu Glu Phe Phe
Arg Ile Phe Asn Arg Ser Ile Asp Ala Phe Lys Asp Phe Val Val
Ala Ser Glu Thr Ser Asp Cys X_n X_n X_n X_n X_n X_n X_n X_n
X_n X_n

wherein n is 0 or 1, X is any naturally occurring amino acid; R_1 is any amino acid except Glu; R_2 is any amino acid except Val; and Q is Lys or Arg to provide human activity, or is Glu to provide murine activity.

The present invention further comprises cDNA sequences that encode hMGF. Human MGF is encoded, for example, by a nucleotide sequence comprising the sequence from nucleotide 112 to nucleotide 516 in Figure 3, or a DNA sequence that hybridizes to a probe defined by the foregoing sequence under conditions of high stringency.

Moderate stringency hybridization conditions, as defined herein and as known to those of skill in the art, refer to conditions described in, for example, Sambrook et al. *Molecular Cloning: A Laboratory Manual*, 2 Ed. Vol. 1 pages 1.101-1.104 (Cold Spring Harbor Laboratory Press 1989). Exemplary conditions of moderate stringency are a prewashing with 5 x SSC, 0.5% SDS, 1 mM EDTA (pH 8.0) and overnight hybridization at 50°C in 2 X SSC. Exemplary severe or high stringency conditions are overnight hybridization at about 68°C in a 6 x SSC solution, washing at room temperature with 6 x SSC solution, followed by washing at about 68°C in a 0.6 x SSC solution.

We initially purified and characterized murine MGF. After obtaining an mMGF cDNA, we obtained the cDNA sequence for hMGF by cross-species hybridization. We have further cloned, sequenced and expressed two variant hMGF cDNAs.

The primary amino acid structure of mammalian MGF may be modified by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like, or by creating amino acid sequence mutants. Such polypeptides are called derivatives. Covalent derivatives of mammalian MGF are prepared, for example, by linking particular functional groups to mammalian MGF amino acid side chains or at the N-terminus or C-terminus of a mammalian MGF polypeptide. Other derivatives of mammalian MGF within the scope of this invention include fusions of MGF or its fragments with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. For example, the fusion polypeptide may comprise a signal (or leader) polypeptide sequence at the N-terminal region of a mammalian MGF polypeptide which co-translationally or post-translationally directs transfer of a mammalian polypeptide from its site of synthesis to a site inside or outside of the cell membrane or wall (e.g., the yeast α-factor leader). Mammalian MGF polypeptide fusions can also comprise fusion to other biologically active proteins or immunoglobulins.

The present invention further includes mammalian MGF polypeptides having altered glycosylation. Mammalian MGF expressed in yeast or mammalian expression systems (e.g., COS-7 cells) may be similar or significantly different in molecular weight and glycosylation pattern than a native mammalian MGF polypeptide. This depends upon the choice of expression system. Expression of mammalian MGF polypeptides in bacterial expression systems, such as *E. coli*, provides non-glycosylated molecules.

Functional mutant analogs of human or mammalian MGF can be synthesized, for example, with inactivated N-glycosylation sites by oligonucleotide synthesis and ligation or by site-specific mutagenesis techniques. N-glycosylation sites in eukaryotic polypeptides are

characterized by an amino acid triplet Asn-Φ-Ω where Φ is any amino acid except Pro and Ω is Ser or Thr. In this sequence, carbohydrate residues are covalently attached at the Asn side chain. Human MGF clone MGF-2D contains four glycosylation sites in the extracellular region of the polypeptide and hMGF-2.4 further contains a fifth glycosylation site in the 28 amino acid alternative exon located in the extracellular region. The glycosylation sites in hMGF-2D begin with the Asn residue at positions 65, 72, 93 and 120. The fifth glycosylation site of human MGF is at position 170. The Δ28 hMGF polypeptide does not contain a fifth glycosylation site. Surprisingly, yeast expression of human MGF hyperglycosylates only the fifth glycosylation site.

Mammalian MGF polypeptides are encoded by multi-exon genes. As exemplified by the various constructs described herein, the present invention includes DNAs and polypeptides corresponding to alternative mRNAs which can be attributed to different mRNA splicing events following transcription, and which share regions of identity or similarity with the cDNAs disclosed herein.

Bioequivalent analogs of mammalian MGF polypeptides or MGF muteins (defined as polypeptides having MGF biological activity and binding to cells expressing c-*kit* proto-oncogenes) may be constructed, for example, by making various substitutions of amino acid residues or sequences, or by deleting terminal or internal residues or sequences not needed for biological activity. Generally, substitutions are made conservatively by substituting an amino acid having physiochemical characteristics resembling those of the replaced residue. Further substitutions may be made outside of the "core" sequence (for human MGF, the core sequence lies between the first the fourth Cys residues) needed for mammalian MGF binding and biological activity. However, the amino acid residue at position 91 is essential for receptor binding and helps to confer species specificity. Subunits of a mammalian MGF polypeptide may be constructed by deleting terminal or internal residues or sequences.

We have purified mMGF to provide a substantially homogeneous polypeptide preparation from a crude protein solution, such as conditioned medium collected from cells expressing MGF. The purification procedure we used is described in Example 1 herein.

The nucleotide sequence and corresponding amino acid sequence of mMGF are illustrated in Figures 1 and 2. These murine sequences were obtained from mMGF-10 and mMGF-94, constructed from +/+ cDNA, that was *Sal* 1 linkered into a *Sal*1 site of vector HAVEo. These isolates were cloned as described in Example 2 herein.

An oligonucleotide probe was created from the nucleotide sequence of isolate 10 used to screen a human cDNA library under moderate stringency conditions. Similar cross species hybridization techniques may be applied to clone new mammalian MGF nucleotide sequences when a particular mammalian sequence (e.g., murine) is known and cDNA or genomic DNA libraries made from cells that would produce MGF polypeptides are available for the desired species.

Expression of MGF Polypeptides

MGF and especially human MGF and analogs thereof can be expressed in a variety of prokaryotic and eukaryotic host systems. Examples of prokaryotic host systems include *E. coli* bacteria and *Staphyllococcus aureus*. Examples of eukaryotic host systems include mammalian cells (COS), Chinese hampster ovary (CHO) and yeast cells such as *S. cerevisiae*.

We have expressed various human MGF polypeptides in a yeast (*S. cerevisiae*) system using a yeast expression vector, pIXY-120 which comprises an ADH2 promoter sequence and the α-factor secretion leader. The ADH2 promoter has been described in Russell et al., *J. Biol. Chem. 258*:2674, 1982, and Beier et al., *Nature 300*:724, 1982. The α-factor leader sequence is often inserted between the promoter sequence and the structural gene sequence. The use of an α-factor leader sequence to improve expression of a particular structure gene has been described in, for example, Kurjan et al., *Cell 30*:933, 1982, and Bitter et al., *Proc. Natl. Acad. Sci. USA 81*:5330, 1984.

The pIXY 120 vector further comprises an *Asp* 718 restriction site at the 5' end and a *Bam* HI restriction site at the 3' end to facilitate insertion of a structural gene. The pIXY 120 expression vector is cut with *Xho* I and an insert comprising a MGF cDNA is ligated into the pIXY 120 vector with *Sal* I restriction enzyme.

Recombinant expression vectors include suitable transcriptional or translational regulatory or structural nucleotide sequences which may be derived from mammalian, microbial, viral or insect genes. Examples of regulatory sequences include transcriptional promoters or enhancers, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and appropriate sequences which control transcription and translation initiation and termination.

Suitable host cells for expression of mammalian MGF or derivatives thereof include prokaryotes, yeast or higher eukaryotic cells under the control of appropriate promoters. Prokaryotes include gram negative or gram positive organisms, for example *E. coli* or *bacilli*. Suitable prokaryotic hosts cells for transformation include, for example, *E. coli*; *Bacillus subtilis*, *Salmonella typhimurium*, and various other species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*. Higher eukaryotic cells include established cell lines of mammalian origin as described below. Cell-free translation systems could also be employed to produce mammalian MGF or derivatives thereof using RNAs derived from the DNA constructs disclosed herein. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described, for example, in Pouwels et al. *Cloning Vectors: A Laboratory Manual*, Elsevier, New York, 1985.

Promoter sequences are commonly used for recombinant prokaryotic host cell expression vectors. Common promoter sequences include β-lactamase (penicillinase), lactose promoter system (Chang et al., *Nature 275*:615, 1978; and Goeddel et al., *Nature 281*:544,

1979), tryptophan (trp) promoter system (Goeddel et al., *Nucl. Acids Res. 8*:4057, 1980; and EP-A- 36,776) and tac promoter (Maniatis, *Molecular Cloning A Laboratory Manual*, Cold Spring Harbor Laboratory, p. 412, 1982). A particularly useful prokaryotic host cell expression system employs a phage λ P_L promoter and a cI857 thermolabile repressor sequence. Plasmid vectors available from the American Type Culture Collection which incorporate derivatives of the λ P_L promoter include plasmid pHUB2 (resident in *E. coli* strain JMB9 (ATCC 37092)) and pPLc28 (resident in *E. coli* RR1 (ATCC 53082)).

Yeast transformation protocols are known to those of skill in the art. One such protocol is described by Hinnen et al., *Proc. Natl. Acad. Sci. USA 75*:1929, 1978. The Hinnen et al. protocol selects for Trp^+ transformants in a selective medium, wherein the selective medium consists of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 μg/ml adenine and 20 μg/ml uracil.

Yeast host cells transformed by vectors containing ADH2 promoter sequence were grown for inducing expression in a "rich" medium. An example of a rich medium is one consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 μg/ml adenine and 80 μg/ml uracil. Derepression of the ADH2 promoter occurs when glucose is exhausted from the medium.

Mammalian or insect host cell culture systems could also be employed to express recombinant mammalian MGF polypeptide or derivatives thereof. Examples of suitable mammalian host cell lines include the COS-7 lines of monkey kidney cells (ATCC CRL 1651) (Gluzman et al., *Cell 23*:175, 1981), L cells, C127 cells, 3T3 cells (ATCC CCL 163), Chinese hamster ovary (CHO) cells, HeLa cells, and BHK (ATCC CRL 10) cell lines. Suitable mammalian expression vectors include nontranscribed elements such as an origin of replication, a promoter sequence, an enhancer linked to the structural gene, other 5' or 3' flanking nontranscribed sequences, such as ribosome binding sites, a polyadenylation site, splice donor and acceptor sites, and transcriptional termination sequences.

Transcriptional and translational control sequences in mammalian host cell expression vectors may be provided by viral sources. For example, commonly used mammalian cell promoter sequences and enhancer sequences are derived from Polyoma, Adenovirus 2, Simian Virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites may be used to provide the other genetic elements required for expression of a structural gene sequence in a mammalian host cell. Viral early and late promoters are particularly useful because both are easily obtained from a viral genome as a fragment which may also contain a viral origin of replication (Fiers et al., *Nature 273*:113, 1978). Smaller or larger SV40 fragments may also be used, provided the approximately 250 bp sequence extending from the *Hind* III site toward the *Bgl* I site located in the SV40 viral origin of replication site is included.

Purification of Recombinant Mammalian MGF

MGF polypeptides may be prepared by culturing transformed host cells under culture conditions necessary to express MGF or derivatives thereof. The resulting expressed polypeptides may then be purified from culture media or cell extracts. MGF or a derivative thereof is concentrated using a commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a purification matrix such as a gel filtration medium. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred (e.g., S-Sepharose® columns).

Finally, one or more reverse-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g., silica gel having pendant methyl or other aliphatic groups, can be employed to further purify MGF. Some or all of the foregoing purification steps, in various combinations, can also be employed to provide a substantially homogeneous recombinant protein. Alternatively, some or all of the steps used in the purification procedure described above for murine MGF can also be employed.

Recombinant protein produced in bacterial culture is usually isolated by initial disruption of the host cells, centrifugation, extraction from cell pellets if an insoluble polypeptide, or from the supernatant if a soluble polypeptide, followed by one or more concentration, salting-out, ion exchange or size exclusion chromatography steps. Finally, RP-HPLC can be employed for final purification steps. Microbial cells can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

Transformed yeast host cells are preferably employed to express MGF as a secreted polypeptide. We made several expression constructs for yeast host cells to secrete various fragments of MGF as a secreted polypeptide. One construct, pIXY 551, encodes an amino acid sequence comprising the first 148 amino acids of human MGF. pIXY 551 was made by mutagenizing pIXY-481 (full length human MGF extracellular domain) to remove 37 amino acids from the C-terminus. PCR reaction conditions allow one to begin with full length (185 amino acids) sequence of the extracellular domain of human MGF and make shorter fragments. pIXY 551 comprises an ADH2 promoter sequence, an α-factor leader sequence, a FLAG sequence, followed by the coding region for the first 148 amino acids of human MGF extracellular domains. We also made shorter versions of human MGF extracellular domain. For example, pIXY 552 comprises the N-terminal 133 amino acids of human MGF

extracellular domain, pIXY 553 comprises the first 119 amino acids of human MGF N-terminal, and pIXY 554 comprises the first 104 amino acids of human MGF extracellular domain.

All of the constructs were transfected into yeast (*Saccharomyces cerevisiae*) strain XV2181 for expression. Yeast were grown to an optimal optical density and supernatants were collected for analysis of protein and human MGF activity in a TF1 cell proliferation assay. When normalized for units per microgram of MGF protein, pIXY 551 and pIXY 490 (Δ28 hMGF) exhibited the highest mean specific activity of 8326 and 6938 units/μg, respectively. By comparison, full-length 185 amino acid MGF produced 4520 units/μg, and another version comprising the first 164 amino acids of the extracellular domain produced 4978 units/μg. Shorter versions, that eliminate the fourth Cys residue, had significantly less biological activity as determined in a TF1 cell proliferation assay. For example, pIXY 553 comprising the first 133 amino acids of the N-terminal sequence had 1143 units/μg of activity while pIXY 553 (119 N-terminal amino acids) and pIXY 554 (104 N-terminal amino acids) did not produce any proliferation activity in the TF1 cell assay when compared to background.

Purification of recombinant human MGF when expressed in yeast supernatants involves three chromatographic steps. Yeast broth from transformed yeast making recombinant human MGF was titrated to pH 3 with a citrate buffer and HCl. The buffered broth is diluted with water and loaded onto a cation exchange column, such as S-Sepharose® (Pharmacia) that has been equilibrated in the citrate buffer. The column is washed with citrate buffer, and MGF is eluted with 50 mM ß-alanine pH 4.0 and a solution of 50 mM sodium acetate pH 5.0. The next step is a reverse phase high performance liquid chromatography (RP-HPLC) with a bonded alkyl column (preferably a C4 column). The column is washed with 0.1% TFA (trifluoroacetic acid) until the pH drops to 2.0. MGF is eluted with a linear gradient of 0-100% acetonitrile in 0.1% TFA. MGF elutes at approximately 50% acetonitrile.

Fractions containing MGF from the reverse phase column were pooled and buffered with 25 mM citrate pH 3.0. MGF was applied to a cation exchange column such as S-Sepharose®, and equilibrated with 25 mM citrate pH 3.0. The column was washed with citrate buffer and eluted with an elution buffer containing 0.5 M NaCl in 50 mM Tris pH 8.0. This step concentrates MGF in the elution buffer.

Administration of Mammalian MGF Polypeptide and Derivative Compositions

The present invention provides methods of using therapeutic compositions comprising an effective amount of MGF in a suitable diluent or carrier. For therapeutic use, purified MGF or a biologically active derivative thereof is administered to a patient, preferably a human, for treatment in a manner appropriate to the indication. Thus, for example, MGF compositions administered to suppress a form of anemia can be given by bolus injection, continuous infusion, sustained release from implants, or other suitable technique. Typically, a MGF

therapeutic agent will be administered in the form of a pharmaceutical composition comprising purified polypeptide in conjunction with physiologically acceptable carriers, excipients or diluents. Such carriers will be nontoxic to patients at the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining a mammalian MGF polypeptide or derivative thereof with buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or dextrans, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with conspecific serum albumin are exemplary appropriate diluents.

The cellular events that lead to blood cell production are controlled by multiple cytokines. Many of these cytokines are produced by marrow stromal cells or by cells that traffic through the marrow compartment. Hematopoietic stem cell development occurs primarily at specific sites within the marrow. Of the hematopoietic stem cells, the human CFU-B1 and HPP-CFC are believed to be the cells responsible for initiating long term hematopoiesis *in vitro*. Each of these cells possesses similar phenotypic properties and a number of biological behavior patterns associated with human stem cells. A variety of *in vitro* assays have been utilized to determine cytokine requirements of these primitive progenitor cells.

When administered alone, MGF (extracellular domain) can augment the proliferation of both myeloid and lymphoid hematopoietic progenitor cells. Most importantly, MGF exhibits potent synergistic activity in conjunction with other colony-stimulating factors that can result in increased colony number and size. More specifically, MGF can synergistically interact with later acting hematopoietic growth factors such as Interleukin-1 (IL-1), Interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), Interleukin-7 (IL-7), and a fusion protein (pIXY-321) that comprises active sites of both GM-CSF and IL-3. pIXY-321 is a GM-CSF/IL-3 fusion protein having$Leu^{23}Asp^{27}Glu^{34}$ hGM-CSF/$Gly_4SerGly_5Ser/Pro^8Asp^{15}Asp^{70}$hIL-3, and is described in U.S. Patent Aplication 07/567,983 filed October 14, 1990, the disclosure of which is incorporated by reference herein.

A bone marrow subpopulation of $CD34^+$ DR^- $CD15^-$ is a model for a group of primitive hematopoietic progenitor cells. When MGF is added to this model of hematopoietic progenitor cells, a modest granulocyte-macrophage colony-stimulating activity but no erythropoietic burst or mixed colony formation activity was evident. Using other models of primitive hematopoietic progenitor cells, MGF has promoted erythropoietic bursts (macroscopic) and mixed colony formation in the presence of EPO.

Most notably, MGF was capable of synergizing with other cytokines, such as IL-3, in promoting hematopoietic cell proliferation. For example, the combination of MGF and the fusion protein pIXY-321 produced a significant increase in the number of CFU-GM and HPP-CFC when compared to the colonies formed with just pIXY-321 alone. Table 1 below presents data from a comparison of CFU-GM, BFU-E, CFU-GEMM, HPP-CFC, and BFU-

MK colony activities stimulated by various cytokines in a population of $CD34^+$ DR^- CD15- human progenitor cells.

Table 1

Cytokines	Colonies per 10^3 $CD34^+$ DR^- $CD15^-$ Cells				
	CFU-GM	BFU-E	CFU-GEMM	HPP-CFC	BFU-MK
None	0.5+/- 0.7	12.5 +/- 3.6	0 +/- 0	0 +/- 0	0 +/- 0
IL-3	23.0 +/- 1.4	31.5 +/- 5.0	2.5 +/- 0.7	0 +/- 0	5.8 +/0 0.3
GM-CSF	56.0 +/- 7.1	24.0 +/- 7.1	3.5 +/- 0.7	1.5 +/- 0.7	2.6 +/- 0.9
IL-3 + GM-CSF	48.5 +/- 5.0	35.0 +/- 14.2	2.5 +/- 0.7	1.5 +/- 0.7	7.9 +/- 1.1
pIXY-321	42.5 +/- 3.6	41.0 +/- 0.0	6.0 +/- 1.4	3.5 +/- 3.1	9.8 +/- 0.7
MGF	18.5 +/- 2.1	14.0 +/- 4.3	0 +/- 0	0 +/- 0	0 +/- 0
MGF + IL-3	--	--	--	--	9.0 +/- 0.4
MGF + pIXY-321	100.5 +/- 3.6	36.5 +/- 3.6	2.5 +/- 2.1	23.5 +/- 10.7	9.8 +/- 0.5

CFU-GM, BFU-E, CFU-GEMM, and HPP-CFC were assayed with about 10^3 $CD34^+$ DR^- $CD15^-$ marrow cells in methyl cellulose containing one unit of human recombinant erythropoietin and enumerated at 21 days, or 28 days for HPP-CFC. Concentrations of cytokine included 2 µg/ml for IL-3 (specific activity 3.5×10^8 CFU/MG protein), 1 ng/ml for GM-CSF (specific activity 2.8×10^8 CFU/MG protein), 10 ng/ml for pIXY-321 (specific activity $1\text{-}2 \times 10^9$ CFU/MG protein), 50 ng/ml for murine MGF (specific activity 10^6 CFU/MG protein). These data were performed in duplicate to obtain the standard deviations indicated. The study was repeated, and similar data were obtained.

MGF alone was capable of sustaining long term myelopoiesis but had a limited affect on megakaryocytopoiesis and erythropoiesis. However, when either pIXY-321 or IL-3 was added with MGF, a profound synergistic effect in promoting progenitor cell expansion of all three hematopoietic lineages was found. The most pronounced effect was seen with the combination of MGF and pIXY-321, which led to a 50-1000 fold increase in progenitor numbers over a 10 week period. MGF was capable of synergizing with IL-3 in promoting megakaryocyte progenitor cell expansion, but was incapable of substantially augmenting maximal proliferative stimulus supplied by pIXY-321 or a combination of GM-CSF and IL-3. Further analyses of these data comparing the effects of MGF alone and in combination with other cytokines showed that although MGF alone had a limited effect upon cellular proliferation of hematopoietic progenitor cells, the presence of MGF within a combination of a later-acting cytokine (e.g., IL-1α, IL-1ß, IL-6, IL-7 , IL-4, IL-3, EPO, LIF, GM-CSF, and a IL-3/GM-CSF fusion protein such as pIXY-321) favored the presence of MGF for the most primitive hematopoietic progenitor cell expansion. Thus, combinations including MGF and other later-

acting cytokines are necessary for maximal proliferation and differentiation of hematopoietic progenitors.

When various cytokines were evaluated in long term cultures of hematopoietic progenitor cells, hematopoiesis was sustained for six to ten weeks in the presence of either IL-3, GM-CSF, pIXY-321, IL-3 plus GM-CSF, or MGF. The combination of IL-3 and GM-CSF was additive, but less than the effect of pIXY-321. Only the combination of MGF plus IL-3 or MGF plus pIXY-321 provided a dramatic synergy, resulting in a 10^3 - 10^5-fold increase in cell numbers over an original 5 x 10^3 cellular inoculum. Therefore, MGF in combination with a later-acting cytokine such as IL-3 or a fusion protein of IL-3 and GM-CSF, promotes cellular proliferation and the presence of MGF in that cytokine combination favors the persistence of a more primitive hematopoietic cell type.

Human megakaryocytopoiesis is regulated by a group of cytokines, including GM-CSF, IL-1α, IL-3, IL-6, and MGF. Megakaryocytopoiesis can be measured by CFU-MK and BFU-MK. Each *in vitro* measurement determines a level of progenitor cell development leading to platelet formation. Defects in megakaryocytopoiesis, on the level of BFU-MK or CFU-MK, ultimately lead to various bleeding disorders due to a failure of proper platelet formation or recruitment. MGF affects multiple stages of megakaryocyte development, and synergistically augments colony stimulating activity of various cytokines, including pIXY-321, GM-CSF, IL-3 and IL-6. MGF in combination with IL-3 potentiates the ability of IL-3 to promote colony formation at each level of progenitor cell development (BFU-MK and CFU-MK). Optimal concentrations of GM-CSF, IL-3, pIXY-321, IL-1α, and IL-3 were roughly equivalent when measuring BFU-MK *in vitro*, and were not further enhanced by the addition of MGF. However, at the level of CFU-MK, MGF synergistically enhanced the ability of pIXY-321, GM-CSF, IL-3 and IL-6 to promote megakaryocyte colony formation *in vitro*. Maximal human megakaryocytopoiesis was achieved with the triple combination of IL-3, GM-CSF and MGF, or the combination of MGF plus a GM-CSF/IL-3 fusion protein such as pIXY-321.

MGF also stimulates erythroid proliferation in the presence of erythropoietin (EPO). In an *in vitro* model with BFU-E derived colonies from patients with sickle cell anemia (a hemolytic anemia) and Diamond-Blackfann anemia (a hypoproliferative anemia), MGF increased the mean number of BFU-E derived colonies with both types of anemia. MGF at a concentration of 50-100 ng/ml can increase fetal hemoglobin synthesis in sickle cell anemia. The stimulation effect of MGF was decreased by concurrent addition of Interferon-γ. The stimulation of BFU-E caused by MGF in the presence of erythropoietin, was evident by measuring both colony number and size, and was significantly increased in a dose-dependent manner according to concentration of MGF. Therefore, the combination of MGF and EPO is useful for treating hemolytic anemias and hypoproliferative anemias.

MGF can also be effective for stimulating lymphoid precursor cells, and influencing early lymphoid development. MGF stimulated thymidine incorporation in day 13 fetal thymus cells. Fetal thymus cells are a source of lymphoid precursor cells. In addition to MGF, both Interleukin-2 (IL-2) and IL-7 can stimulate significant tritiated thymidine incorporation in day 13 fetal thymus cells. The effects of the combination of MGF and IL-2 were additive, while the effects of MGF plus IL-7 were greater-than-additive. When compared with background, day 13 fetal thymus cell expansion after 9 days of incubation with cytokine, were three-fold higher than background for MGF, three-fold higher than background for IL-7, and 103-fold higher than background for the combination of MGF and IL-7. Fetal thymus cells that were expanded by the combination of MGF and IL-7 were $CD3^-$ $CD4^-$ $CD8^-$ cells. The combination of MGF plus IL-7 stimulated pre B cell colonies two to four-fold above that seen with IL-7 alone. These data illustrate the importance of combining MGF with IL-7 to augment and proliferate early lymphoid cells.

MGF may have further function in organization of the neural tube and brain. More importantly, MGF mediates proliferation and differentiation of fetal neural tube cells to promote development of fetal brain and brain in newborns. Moreover, MGF effects melanocyte function to regulate differentiation and proliferation of melanocytic functions.

The following examples are for purposes of illustration and not by way of limitation.

EXAMPLE 1

PURIFICATION OF MURINE MGF

This example describes purification of murine MGF to homogeneity for amino acid sequencing. 45 liters of conditioned medium was obtained from normal WCB6F1 +/+ cells grown in RPMI 1640 supplemented with 10% fetal calf serum. The conditioned medium was first acidified to pH 2.75 by adding concentrated HCl and buffered with Na citrate, pH 3.25, to a final concentration of 5 mM Na citrate. The acidified conditioned medium was applied to an S-Sepharose® Fast Flow (Pharmacia) column equilibrated with 0.1 M NaCl 5 mM Na citrate, pH 3.25. After non-bound proteins cleared the column, bound protein was eluted with 0.1 M EPPS (N [2-hydroxyethyl]-piperazine-N'-[3-propane-sulfonic acid] Sigma Chemical Co.), pH 8.5. The eluted protein pool was diluted 1:3 with water and loaded on a DEAE Sephacel (Pharmacia) column equilibrated with 10 mM EPPS, pH 8.5. Protein bound to the column was eluted with a salt gradient (0 - 0.5 M NaCl in 10 mM EPPS, pH 8.5). Fractions were collected and bioassayed using MC6 cells in a biological assay as described herein. Fractions having an activity of at least about 10 units per ml were deemed "active" fractions and were pooled for further purification procedures.

The protein pool was diluted with 2M ammonium sulfate 20 mM Tris HCl, pH 7.5, and centrifuged to remove precipitated proteins. The pooled protein was then applied to a Phenyl Sepharose® CL-4B (Pharmacia) column equilibrated with 2 M ammonium sulfate 20 mM Tris HCl, pH 7.5. Bound proteins were eluted using a reverse gradient of 2 M - 0.5 M ammonium sulfate in 20 mM Tris HCl, pH 7.5. Fractions were collected, dialyzed against PBS (using a 6000-8000 dalton cut-off membrane), and assayed for MGF biological activity using the MC6 cell assay described herein. Fractions containing MGF were pooled for further purification procedures.

Pooled active fractions from the Phenyl Sepharose purification were buffer exchanged with 15 mM $CaCl_2$ 20 mM Hepes, pH 7.5, and concentrated about 10 to 12 times using an Amicon stirred cell with a YM 10 membrane. The concentrated Phenyl Sepharose pool was passed over a Matrex® gel Blue A (Amicon Corp.) column washed with 5 mM $CaCl_2$ 20 mM Hepes, pH 7.5, to remove additional contaminating proteins that bind to the Blue A column. The Blue A pool was acidified by addition of trifluorocetic acid (TFA) while vortexing to a final TFA concentration of about 0.1% (v/v) and filtered through a .022 μ cellulose acetate filter. The acidified protein pool was pumped directly onto a Radial-Pak Vydac® C-4 column (15 μ particle size) pre-equilibrated with 0.1% TFA in water at a flow rate of about 0.8 ml/min. Bound proteins were eluted using a 1%/min linear gradient of acetonitrile (0.1%, v/v TFA). One minute fractions were collected, neutralized with a Tris buffer, and bioassayed for MGF biological activity.

Active fractions were pooled, and the protein pool was concentrated by rotary evaporation to about 10% of its original volume. The concentrated protein pool was applied to

an Aquapore® Butyl C-4 column (7 μ particle size) pre-equilibrated with 0.1% TFA in water, at a flow rate of about 0.2 ml/min. Protein bound to the column was eluted with a .05%/min linear gradient of acetonitrile (0.1%, v/v TFA). Fractions were collected, neutralized, and bioassayed for MGF activity.

Active fractions were pooled for SDS-PAGE. Protein fractions exhibiting MGF activity were collected from 2 runs of the purification protocol described above and concentrated to dryness by rotary evaporation. The protein was resuspended in gel sample buffer and heated to 110°C for five minutes. The protein was then separated using a 10% Laemli gel and transferred onto a PVDF membrane (Pro Blot, Applied Biosystems) using constant current, 60V setting, for 1.0 hour. Three protein bands were visualized by staining the PVDF membrane for five minutes with 0.1% Coomassie Blue in 10% acetic acid, 50% methanol.

The membrane was destained in the acetic acid/methanol solution without Coomassie blue stain, washed extensively with HPLC water, and dried. Protein bands were excised with a razor and placed onto a pre-cycled filter for a model 477 protein sequencer (Applied Biosystems, Foster City, CA), for N-terminal amino acid sequencing.

EXAMPLE 2

CLONING A MURINE MGF cDNA

Polyadenylated RNA was prepared from +/+ cells (described in Boswell et al. *Blood 70*:167a 1987) and cDNAs were prepared using standard techniques. The +/+ cell line produces murine MGF. cDNA ends were adapted with *Sal* 1 adapters (Haymerle et al. *Nucleic Acid Res. 14*:8615-24 1986):

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5'-TCGACTGGAACGAGACGACCTGCT-3'
    3'-GACCTTGCTCTGCTGGACGA-5'
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and cloned into vector HAVEo. A pool consisting of approximately 5000 individual plasmid-containing isolates was plated and screened by hybridization to a DNA fragment. The DNA fragment was prepared using polymerase chain reaction (PCR) amplification of murine MGF sequences from +/+ and LDA11 cell line cDNA as follows.

The sequence of the N-terminal 28 amino acids of purified murine MGF was used to design synthetic oligonucleotide primers for PCR amplification of murine MGF-cDNA clones from a murine library described below. The first five amino acids of the N-terminus (Lys Glu Ile Cys Gly) were used to design one primer, 5'-CGCCCGGGAA(G/A)GA(G/A)AT(A/C/T)TG(T/C)GG-3', a degenerate mixture coding for all possible codon usages of the first five amino acid residues, omitting the third position of Gly and containing an additional eight bases coding for a *Sma*1 recognition site and a 5' CG

clamp. The amino acid sequences of the mouse mature N-terminus 23-28 (Pro Asn Asp Tyr Met Ile) were used to design a second primer, a degenerate mixture coding for a complement of all possible codon usages of amino acids 23-28, omitting position 3 of Ile and containing an Asp 718 recognition site and a 5' CGC clamp:
5'-CGCGGTACCATCAT(G/A)TA(G/A)TC(G/A)TT(G/A/C/T)GG-3'.

Polyadenylated RNA from mouse secretor cell lines +/+ and LDA11, stimulated under a variety of conditions (LDA11, 45% McCoy's medium, 45% HL-1 medium, 10% PBS, 6 hrs; LDA11, HL-1 medium, 28 hrs; LDA11, HL-1 medium, 5.5 hrs; +/+, Opti-MEM medium 9 hrs), was used as separate templates for first strand cDNA synthesis. A portion of first strand cDNA reactions was added to commercially available PCR reaction mixes containing the oligonucleotide primers. This mixture was subjected to 30 cycles of PCR amplification.

Following amplification, samples were purified and subjected to agarose gel electrophoresis. This yielded a 100 base pair DNA fragment that was excised from gel lanes from four separate reactions involving +/+ cells and LDA11 cells. The 100 base pair DNA fragment was purified using an Elutip-D® column (Schleicher & Schuell, Keene, NH), cloned into pBluescript® SK (Stratagene, La Jolla, CA) and used for dideoxy DNA sequencing.

We prepared a hybridization probe by random prime labeling of the purified 100 base pair DNA fragment and used the hybridization probe to screen a portion of the plasmid library containing cDNA inserts prepared from +/+ polyadenylated RNA. Several positive clones were further characterized, including MGF isolate 4, isolate 10, and isolate 94. Isolate 4 contained sequence information for the coding region minus the last four amino acids of the C terminus. Isolate 10 contained the entire coding region for the mouse polypeptide and is illustrated in Figure 2. Isolate 94 has a 16 amino acid deletion in the extracellular domain compared to isolates 4 and 10.

EXAMPLE 3

IDENTIFICATION OF MGF AS THE LIGAND FOR C-*Kit*

In order to demonstrate that MGF is the ligand for c-*kit*, murine MGF purified as described in Example 1 was bound to cells expressing c-*kit*. The resulting c-*kit*/MGF complexes were covalently cross-linked and immunoprecipitated using antibody specific for c-*kit*. Radioactivity was recovered in the immunoprecipitated fraction.

We first cloned murine c-*kit* cDNA using polymerase chain reaction from poly A^+ selected mRNA isolated from MC6 cells (an MGF responder cell line). A 3.0 kilobase cDNA was sequenced and compared to the published murine c-*kit* sequence (Qui et al. *EMBO J.* 7:1003 1988). Our clone had two amino acid changes from the published sequence, including an alanine instead of a glutamic acid at position 207 and a glycine instead of an alanine at amino acid 777 (numbered according to Qui et al.).

We generated anti-peptide antisera to our murine c-*kit* protein. C-terminal peptides corresponding to the last 10 amino acids of the published sequences, amino acids 76-89 and amino acids 730-743 were synthesized. The synthesized peptides were conjugated with ovalbumin and injected into rabbits. Serum used for immunoprecipitation experiments was obtained from the rabbits after an initial injection and four subsequent boosts. All three antisera, corresponding to the three different peptides, precipitated a protein of 145 kDa from transfected Rat-2 cells stably expressing c-*kit*, whereas no proteins were precipitated with preimmune serum or from non-transfected control Rat-2 cells.

Radiolabeled purified mouse MGF was added to MC6 (responder cell line expressing c-*kit* as described herein) and 32D cells (non-responder cell line that does not express c-*kit*) with and without excess unlabeled MGF. MGF was cross-linked to its specific cell surface receptor by the cross-linking agent BS3. Cell lysates were immunoprecipitated with the specific antisera described above, which recognize the C-terminal portion of c-*kit*, then separated on an SDS-PAGE gel and subjected to autoradiography. Cross-linked receptor-ligand complexes were specifically immunoprecipitated by anti-c-*kit* antisera only from MC6 cell lysates incubated in the absence of cold-competitor and had an approximate molecular weight of 175-180 kDa as determined by an autoradiogram of cross-linked receptor (c-*kit*) ligand (in MGF) complexes (See Figure 6). Preimmune sera did not immunoprecipitate this complex and binding in the presence of excess unlabeled MGF provided ro cross-linked complexes detectable in autoradiography. No evidence of MGF binding to 32D cells was seen.

When one subtracts the molecular weight of the c-*kit* protein from the total molecular weight of the complex, the suggested molecular weight of the ligand is from about 30 kDa to about 35 kDa. These data are consistent with the molecular weight determination of recombinant MGF.

EXAMPLE 4

CROSS HYBRIDIZATION OF A MOUSE MGF PROBE TO A HUMAN LIBRARY

A probe was prepared from a cDNA insert of MGF isolate 10 (an approximately 2 kb *Sal*I fragment) by random prime labeling with α-^{32}P-dCTP to a specific activity of greater than 5×10^5 dpm/ng. The probe was hybridized to human RNAs on.Northern blots in buffer (50% formamide, 0.8 M NaCl, 0.02 M PIPES pH 6.4, 2 mM EDTA, 0.5% sodium dodecyl sulfate (SDS), 100 µg/ml salmon sperm DNA and 5×10^5 dpm/ml of labelled DNA) at approximately 42°C for greater than 20 hours. Hybridization was followed by extensive washing in 1 X SSC (15 mM trisodium citrate, 165 mM NaCl), 0.1% SDS at approximately 55°C.

We found specific bands by autoradiography of probed and washed blots containing polyadenylated mRNA from the human cell lines RAJI (B cell lymphoma), WI26-VA4 (SV40-transformed lung fibroblast), HeLa (ovarian carcinoma), T24 (bladder carcinoma), IMTLH

(SV40-transformed stromal line), IE9 (a subclone of IMTLH), peripheral blood T cells (PBT) stimulated with PHA and PMA, PBT grown 6 days in IL-2 and OKT3 MAb then stimulated with ionomycin and PMA, and PBT grown for six days in IL-2 and OKT3 then stimulated with concanavalin A and PMA.

We observed cross hybridization to specific bands in human genomic DNA (Southern blots) when the MGF isolate 10 probe was added in hybridization buffer containing 30% formamide, with hybridization conditions as described in this example at approximately 42°C, followed by extensive washes in 0.5 X SSC, 0.1% SDS at approximately 63°C.

We have used an MGF-10 probe in cross hybridization in a buffer containing 40% formamide with hybridization conditions as described in this example at approximately 37°C followed by extensive washes in 1 x SSC, 0.1% SDS at approximately 55°C to screen a PBL library. We isolated MGF-positive human clones from this library.

These cDNAs contain only a portion of the 3' MGF coding region, based on a comparison with mMGF. Full length hMGF coding cDNAs were isolated by polymerase chain reaction (PCR) amplification of hMGF cDNAs from HeLa mRNA using oligonucleotide primers designed to specifically amplify hMGF coding cDNAs. The partial cDNA sequence derived from the PBL library clone was used to design a 3' non-coding region oligonucleotide primer (5'-AATGTTACCAGCCAATGTACG-3') which is complementary to sequences located 30 bp downstream of the end of translation in the hMGF cDNAs (nucleotides 878-898 of hMGF-2.4, Figure 3). The 5' non-coding oligonucleotide primer (5'-GGGCTGGATCGCAGCGC-3') was derived from the mMGF 5' non-coding region (nucleotides 122-138 of MGF-10, Figure 1). These oligonucleotides were used to amplify hMGF cDNAs from HeLa in a reaction buffer containing 50 mM KCl, 10 mM Tris pH 8.3, 0.01% gelatin,1.5 mM $MgCl_2$ 200 µM dNTPs, 1 µM each oligonucleotide primer, 2.5 units Taq polymerase, and approximately 20 ng first strand HeLa cDNA. Reactions were performed on an Ericomp TwinBlock® temperature cycler (Ericomp, San Diego, CA) for 35 cycles of 94 °C for 30 seconds, 45 °C for 45 seconds and 72 °C for 60 seconds, followed by an additional 5 minutes of extension at 72 °C. Agarose gel electrophoresis of the products of PCR amplification showed that at least four distinct sizes of cDNA were isolated. The most abundant size class migrated in the gel at approximately 850 bp. The second most abundant size class migrated at approximately 800 bp. These cDNA products were subcloned and sequenced. Isolate hMGF-2.4 corresponds to the most abundant cDNA from the PCR amplification. Isolate hMGF-2D corresponds the the next most abundant cDNA.

Analysis of mouse MGF and human MGF shows approximately 89% nucleotide sequence identity and approximately 83% amino acid sequence identity between human and murine MGF sequences.

We can also use the isolate 10 clone (mMGF-10) as a cross-hybridization reagent at approximately 63°C in buffers containing 50% or less formamide, followed by washes in 2 X

or less SSC at 50°C to 63°C. DNA probes can also be prepared by nick translation of mouse MGF cDNA by polymerase chain reaction (PCR) amplification using MGF-specific primers and radiolabeled d NTPs, and by preparation of radiolabeled cDNA using synthetic MGF RNA as a template. The DNA probes can be hybridized in hybridization solutions containing 50% or less formamide at temperatures ranging from 37°C to 50°C, followed by washes at 50°C or above in 2 X SSC or less.

EXAMPLE 5

EXPRESSION OF MGF

This example illustrates the expression of full length murine MGF in COS cells (derived from African Green Monkey kidney cells). Isolate 10 and isolate 4 were transfected into COS cells using standard techniques, such as those described in Cosman et al. *Nature 312*:768 (1984). The transfected COS cells were seeded into 96 well plates. Responder cells (e.g., MC6 cells) that proliferate in response to MGF were added to the wells and incubated overnight. Tritiated thymidine was added to the wells at a concentration of 0.5 μCi/well. COS cells transfected with HAVEo empty vector were incubated in parallel as a control. A positive control (not shown) consisted of COS cells transfected with HAVEo engineered to express a murine IL-4 cDNA. The MC6 responder cell line also proliferates in the presence of IL-4. Proliferative activity significantly increased ($p < 0.001$) in the presence of MGF when compared to empty vector controls. For example, clone 4 induced proliferative activity in MC6 cells of about 28,000 cpm compared to about 13,000 for an empty vector control. Both clones 10 and 4 provided mMGF polypeptides with about 13,000 cpm compared to about 4,000 for empty vector control.

We also expressed the extracellular region of murine MGF in COS cells. We prepared a *Sst* 1 - *Bgl* 1 fragment encoding the extracellular portion of mMGF through Ala 179 (Figure 2). This is five amino acids N-terminal from the transmembrane region. This fragments was blunted with T4 polymerase and inserted into a vector pDC402 to generate the plasmid PDC402/sMGF-1. Fifteen additional amino acids (Val Asp Gly Pro Cys Gly Arg Tyr Arg Ser Thr Arg Phe Asp Val) and a termination codon were provided by the vector downstream of the coding site or toward the C-terminus of the truncated polypeptide. COS-7 cells were transfected with either the vector containing the truncated cDNA, with vector containing a murine interleukin-4 cDNA (pDC402/mIL-4), or with vector lacking an insert. The transfected COS cells were metabolically labeled with ^{35}S-methionine and ^{35}S-cysteine. Supernatants were analyzed by SDS-PAGE for size determination of the secreted polypeptide and found a 33 kDa protein (Extracellular region plus glycosylation). Supernatants were also tested for MGF biological activity using the MC6 proliferation assay described herein. The secreted extracellular portion of murine MGF was found to possess MGF biological activity.

Another soluble version of MGF, containing only the extracellular domain of the polypeptide (amino acids 1-185, Figure 2), was constructed in a yeast expression system. Synthetic oligonucleotide primers were used to amplify the entire extracellular coding domain of murine MGF. A 5' primer (5'-AATGTTTACCTTTGGATAAAAGAGACTACAAGGACGACGATGACAAGAAGGAGATCTGCGGGAATCCTGTG-3') encoded an alpha factor leader and an antigenic octapeptide (FLAG®), Hopp et al. *Bio/Technology* 6:1204 (1988) fused in-frame with the mature N-terminus of MGF. The 3' oligonucleotide (5'-ATATGGATCCCTAGTCTTCAGGGGCCTTTGCGGCTTT-3') created a termination codon following Asp-185 (Figure 2), just upstream from the transmembrane region. This DNA fragment was PCR-generated and ligated into a yeast expression vector that directs secretion of the recombinant product into yeast medium (Price et al. *Gene 55*:287 (1987). There was one alteration in the sequence of the PCR-generated DNA, which converted asparagine 169 to serine, eliminating one of the four potential sites for N-linked glycosylation. The resulting murine MGF fusion protein was purified from yeast broth by affinity chromatography using procedures described in Hopp et al., *supra*.

Biological activity of recombinant yeast-made MGF and purified murine MGF was compared in the MC6 proliferation assay. Both recombinant MGF and purified murine MGF stimulated proliferation of MC6 cells in a dose-response fashion. There was some reduction of thymidine incorporation seen at the highest concentration of recombinant MGF, but this may have been due to the presence on non-specific inhibitors of cellular proliferation in the yeast broth. When the yeast broth was purified, the maximum thymidine incorporation by MC6 cells was identical to that observed with natural murine MGF. These data were repeated with murine MGF-responsive cell lines H7 and FDC-P2-1D instead of MC6 cells. Again, the pattern of responsiveness to the recombinant yeast-derived fusion protein was identical to that derived from the purified murine natural MGF polypeptide.

EXAMPLE 6

EXPRESSION OF HUMAN MGF IN YEAST

This example describes yeast expression of human full length extracellular domain MGF and Δ28 hMGF, which lacks a 28 amino acid sequence in the extracellular domain. We used two yeast expression constructs, pIXY-528 and pIXY-490. Both constructs were made by inserting a human MGF cDNA into a pIXY-120 vector comprising a ADH2 promoter sequence, an α-factor leader sequence, and a FLAG® octapeptide sequence for purification. pIXY-528 encoded a FLAG® full length human MGF extracellular domain, whereas pIXY-490 encoded a FLAG® Δ28 human MGF extracellular domain. Yeast (*Saccharomyces cerevisiae*) host strains XV2181 and YNN281 were transformed with pIXY-528 or pIXY-

490. Material made from pIXY-528 showed a high degree of hyperglycosylation and a lower level of protein expression than the material made from pIXY-490. A controlled experiment was conducted utilizing host strain XV2181 and an ELISA assay for total MGF expression in yeast supernatants. The yeast were grown to the same optical density and supernatants were purified. pIXY-528 produced approximately 50 mg/liter of human MGF whereas pIXY-490, grown under identical conditions with the same expression vector in the same yeast host strain, produced five times more protein, or about 250 mg/l. Further examination of the material produced by pIXY-490 shows virtually no hyperglycosylation judging by two bands visible on a FLAG Western blot. The material produced by pIXY-528 was hyperglycosylated with at least five bands visible on a FLAG Western blot.

Full length human MGF has five potential N-linked glycosylation sites, whereas Δ28 human MGF has four. Further, multiple serine residues have been removed from the Δ28 version. It is believed that the series of serine residues function as O-linked glycosylation sites. Removal of the fifth N-linked glycosylation site and serine-rich domain in the Δ28 version of human MGF, surprisingly, resulted in reduced glycosylation, greater MGF biological activity, and approximately five times greater amounts of protein expression when grown under identical conditions.

EXAMPLE 7

BINDING TO EXPRESSED c-*Kit*

This example illustrates that MGF is the ligand for c-*kit*. The experiment described in Example 3 was repeated, except, the c-*kit* clone was transfected into COS cells (along with an empty vector control) and the transfected COS cells were used instead of MC6 responder cells. The procedures described in Example 3 were followed. We found precipitation of iodinated MGF cross-linked to c-*kit* only in those COS cells transfected with c-*kit*. These data provide further evidence that MGF is a ligand for c-*kit*.

EXAMPLE 8

MGF STIMULATES COLONY FORMATION *IN VITRO*

This example compares colony formation induced by various cytokines and controls. Two assays were employed. In the first assay, granulocyte-macrophage colony- and cluster-forming cells (CFU-GM) were assayed by a procedure substantially similar to that described in Williams et al. *Exp. Hematol. 15*:243 (1987). Briefly, bone marrow cells were suspended in 0.5 ml of 0.3% agar (Difco, Detroit MI) or 0.4% agarose (FMC, Rockland, ME) culture medium containing McCoy's 5A medium supplemented with essential and nonessential amino acids, glutamine, serine, asparagine, and sodium pyruvate (Gibco) with 20% fetal bovine

serum. Quadruplicate cultures were incubated for seven days in a fully humidified atmosphere of 5% CO_2 in air. Colonies (> 50 cells) and clusters (3-49 cells) were counted with an inverted microscope at 32 X.

In the second assay, erythroid burst-forming units (BFU-E) and multipotential colony-forming cells (CFU-GEMM) were assayed by a procedure described in Williams et al., *supra*. Briefly, duplicate 35 x 10 mm cultures were stimulated with 2 units per ml of recombinant human erythropoietin (Hyclone), 0.1 mM hemin (Kodak), and 1000 U/ml IL-3 as a source of burst promoting activity. Cultures were incubated for seven days in a fully humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. Maximal colony formation by murine BFU-E and CFU-GEMM was observed on day 7 using the foregoing culture conditions. BFU-E and CFU-GEMM were scored using an inverted stage microscope at 80 X on the basis of hemoglobinization of erythroid elements, producing a characteristic red color, and the absence or presence of myeloid elements for the former and latter, respectively.

Using the foregoing assay, we compared the colony-forming activity of murine IL-3, human EPO, murine MGF at a 1:100 dilution, IL-3 plus EPO, and MGF plus EPO. The results are set forth in the following table:

Table 2

Colony Forming Activity of Cytokines Including MGF

Cytokine	BFU-E/10^5 cells	CFU-GEMM/10^5 cells
none	0	0
IL-3	0	0
EPO	13 +/- 1	1 +/- 1
MGF (1:100)	0	0
IL-3 + EPO	21 +/- 1	8 +/- 1
MGF + EPO	16 +/- 2	8 +/- 1
MGF + IL-3	16 +/- 2	8 +/- 1

These data indicate that MGF stimulates erythroid and primitive mixed colony formation. In another experiment we compared CFU-GM activity of MGF and GM-CSF versus no cytokine control. MGF had significant CFU-GM stimulatory activity (more than GM-CSF) with a stimulation index over background above 50. Moreover, as shown in Figure 5, the BFU-E and CFU-GEMM activities of MGF increase with increasing doses of MGF. This is in contrast to EPO (erythropoietin) whose BFU-E activity remains constant across the dose range.

EXAMPLE 9

MGF PLUS IL-3 SYNERGISTICALLY STIMULATES PRIMITIVE STEM CELLS

Sorted hematopoietic stem cells can be further sorted for metabolic activity. A mitochondrial stain rhodamine 123 stains mitochondria and allows sorting of "bright" cells that are metabolically active and "dull" cells that are not metabolically active. The sorting procedure leading to bright and dull cells is a modification of the procedure described in Visser et al. *Blood Cells* 14:369 (1988). Briefly, a low density (< 1.055 g/cm^3) bone marrow cell fraction from untreated animals (e.g., mice) is isolated by spinning the cells over a discontinuous density gradient of metrizamide. During centrifugation, the cells are stained with WGA/Tx Red. The WGA/Tx Red stained low density cells are next stained with 15-1.4.1/FITC. Stem cells are selected on a cell sorter by detecting for Tx Red$^+$ cells, setting a FLS window on the WGA$^+$ cells to exclude the smallest and largest cells and cell aggregates, selecting cells having the lowest SSC when looking at the SSC intensity distribution of the cells in the TxR/FLS window, and selecting for negative cells when looking at the 15-1.4.1 (FITC) fluorescence distribution of cells in the Tx Red/FLS/SSC window. After the sort, WGA/Tx Red conjugates on removal and the cells are stained with Rhodamine 123. The cells are again sorted in the same FLS and SSC windows as before on the basis of their Rh123 distribution into Rh123 dull and Rh123 bright fractions. Dull cells have marrow repopulating activity and brights do not. Bright cells contain most of the *in vitro* colony forming cells.

Bright cells and dull cells were compared for proliferative activity in a tritiated thymidine incorporation assay. Approximately 1000 cells were incubated in the presence of mMGF, mIL-3 or mMGF plus mIL-3. Table 3 below shows the synergistic proliferation activity of MGF plus IL-3.

Table 3

	CPM	
GROWTH FACTOR	DULL	BRIGHT
Medium control	668 (139)	380 (87)
mMGF (33 U/ml)	2569 (874)	5166 (748)
mIL-3 (100 ng/ml)	9371 (770)	34472 (13226)
IL-3 + MGF	35132 (3098)	91112 (33475)

These data show the synergistic effect on stem cell proliferation in both the bright and dull populations with the combination of MGF plus IL-3. These data were initially obtained with purified murine MGF as described herein and later repeated with purified recombinant yeast-derived murine MGF as described herein. Both sources of murine MGF provided the same results.

The proliferation assay was repeated with MGF, IL-3 and the combination of MGF and IL-3 as described in this example, except proliferation was measured by viable cell counting with trypan blue exclusion. The same synergistic activity of the combination was seen with both bright and dull populations of stem cells. These data are shown in Figure 6a.

We examined the activity of mMGF, mIL-3 and the combination of both in a spleen-colony forming assay with primitive stem cells (CFU-S). The assay looks for functional activity of a population of primitive hematopoietic cells. The procedure is described in Visser et al. *supra*. Briefly, mice are lethally irradiated to wipe out bone marrow cells. Cells (i.e., dulls or brights) are injected into each irradiated mouse. After 14 days, the mice are sacrificed and the spleens are examined and counted for macroscopic colonies on the surface of the spleen. Each colony is derived from a single primitive hematopoietic colony-forming cell (CFU-S). The injected primitive cells were either control (freshly isolated), or treated for three or seven days with mMGF, mIL-3, or the combination of mMGF and mIL-3. The concentrations of mMGF (50 U/ml) and mIL-3 (250 ng/ml) were plateau amounts in colony assays and proliferation assays. A Unit (U) of murine MGF is the amount of mMGF that stimulates half maximal tritiated thymidine incorporation by the MC6 cell line.

Both mMGF and mIL-3 when used alone maintained CFU-S activity for their three days of incubation. When mMGF and mIL-3 are incubated for seven days with the primitive dull cells, only mMGF but not mIL-3 maintains CFU-S activity of the primitive dull cells. When using bright cells, mMGF cannot maintain CFU-S activity at either 3 or 7 days, but mIL-3 can maintain CFU-S activity for both 3 and 7 days. Surprisingly, the combination of mMGF and mIL-3 achieved an expansion of CFU-S with the primitive dull cells. The activity of the combination to cause an expansion of the population of the most primitive stem cells has never been seen before with this model. These data are shown in Figure 6b.

Moreover, we and others have examined all possible combinations of known hematopoietic factors without achieving an expansion before. Accordingly, the combination of MGF and IL-3 provides a synergistic effect to cause activation and proliferation of the most primitive population of stem cells. Thus the therapeutic combination of MGF and IL-3, preferably conspecific MGF and IL-3, is useful for treating anemias and other forms of bone marrow failure that require activation and proliferation of the most primitive stem cell populations. This combination will be most effective for treating aplastic anemia, bone marrow transplantation, and reduced hematopoietic counts caused by any reason. Moreover, as MGF has demonstrated proliferative activity in a population of the most primitive stem cells, it will be effective in combination with another one or combination of hematopoietic factors have have proliferative activity for more developed cells in the hematopoietic system. these other hematopoietic factors include, for example, GM-CSF, G-CSF, IL-3, EPO, IL-1α, IL-1β, IL-7, LIF, IL-6 and combinations thereof.

EXAMPLE 10

PREPARATION OF MONOCLONAL ANTIBODIES TO hMGF

Preparations of hMGF are used to generate monoclonal antibodies against hMGF using conventional techniques. Examples of sources of hMGF for use as an immunogen include, for example, purified recombinant MGF, human MGF, or transfected COS cells expressing high amounts of MGF as described in Example 5 herein. An example of a convention technique to generate monoclonal antibodies is described in United States Patent 4,411,993. Monoclonal antibodies generated by these techniques are likely to be useful in establishing convenient assays for MGF, or for blocking the effects of MGF *in vitro* or *in vivo*.

Mice are immunized with an MGF immunogen emulsified in complete Freund's adjuvant and injected subcutaneously into Balb/c mice at amounts ranging from 10-100 μg. Ten to twelve days later the immunized animals are boosted with additional immunogen emulsified in incomplete Freund's adjuvant. The animals are periodically boosted on a weekly or biweekly immunization schedule. Serum samples are taken periodically for assay of antibody titer, such as by dot-blot or ELISA (enzyme-linked immunosorbent assay). Serum samples may be obtained by retro-orbital bleeding or by tail-tip excision. Once an appropriate antibody titer is detected in positive animals, the positive animals a injected intravenously with MGF in saline. Three or four days after the saline injection, the animals are sacrificed and spleens harvested to splenocytes.

Splenocytes are fused to a murine myeloma cell line, such as NS1 to generate hybridoma cell lines. The hybridoma cell lines are plated in multiple microtiter plates in a HAT (hypoxanthine, aminopterin and thymidine) selective medium to inhibit proliferation of non-fused cells, myeloma hybrids and spleen cell hybrids.

An ELISA techniques can be used to screen for positive hybridoma clones by assaying for reactivity with MGF. Such techniques are described, for example, in Engvall et al. *Immunochemistry* 8:871 1971 and in United States Patent 4,703,004. Anti-MGF monoclonal antibodies are made, for example, in mouse ascites by injecting positive hybridoma clones into peritoneal cavities of syngeneic Balb/c mice. The ascites fluid is purified by standard techniques, such as ammonium sulfate precipitation followed by gel exclusion chromatography, or by affinity chromatography by binding the monoclonal antibody to Protein A (*Staphylococcus aureus*).

EXAMPLE 11

GENERATION OF MONOCLONAL ANTIBODIES TO MURINE MGF BY IMMUNIZATION WITH MGF-BEARING CELLS

A cDNA encoding mMGF is prepared and inserted into C127 cells (ATCC No. CRL 1616) as described in Dower et al. *J. Immunol.* 142:4314-20 1989. Briefly, this procedure

adds the entire bovine papilloma virus genome linearized at a *Bam* HI site to pDC201 containing mMGF cDNA to form a plasmid BX8. BX8 is transfected into C127 cells along with plasmid PSV2 Neo at a ratio of 10:1 (BX8 to PSV2 Neo). Transformed cells expressing mMGF are selected.

Approximately 10^6 selected transformed C127 cells are used to immunize each Lewis rat. Immunization is by intraperitoneal injection three times at three week intervals. Antibody titer is determined by an appropriate assay. Once an appropriate antibody titer is achieved, the rat is boosted by approximately 2×10^6 selected transformed C127 cells and sacrificed three days later. Spleen cells are harvested from the rat and fused with mouse myeloma cells (NS-1, ATCC No. TIB 18) at a 4:1 ratio with 50% polyethylene glycol (PEG MW 1500, EM Reagents, Stuttgard, Germany) using standard fusion procedures. Spleen cells are harvested from the rat and fused with mouse myeloma cells (NS-1, ATCC No. TIB 18) at a 4:1 ratio with 50% polyethylene glycol (PEG MW 1500, EM Reagents, Stuttgard, Germany) using standard fusion procedures. The fused cells are called hybridoma cells.

Hybridoma cells are seeded into well plates at a density of 2×10^5 cells per well in a volume of 200 µl media. Hybridoma cells are screened for monoclonal antibody production to MGF by an assay procedure, such as an ELISA assay.

EXAMPLE 12

GENERATION OF MONOCLONAL ANTIBODIES TO MGF BY IMMUNIZATION WITH VACCINIA BEARING MGF

cDNA containing the entire coding region of MGF or the extracellular region of MGF is prepared as described herein. The cDNA is inserted into the *Sma*I site of vaccinia virus (VV) plasmid expression vector pSC11 (available by license from the U.S. Dept. of Commerce, National Technical Information Service, 5285 Port Royal Rd, Springfield, VA 22161) utilizing a known methods, such as one described in Chakrabarti et al. *Mol. Cell Biol.* 5:3403-09 1985. Transformed VV are visualized by blue plaques. VV from blue plaques are selected and used to infect a host cell, such as CV-1 (ATCC CCL 70) or HeLa (ATCC CCL 2). Infected cells are tested for expression of MGF. Recombinant VV from a positive plaque was purified using conventional techniques, such as those described in Chakrabarti et al. *supra* and Elango et al. *Proc. Natl. Acad. Sci. USA* 83:1906-10 1986.

Lewis rats are immunized by intradermal injection with approximately 10^8 plaque forming units (pfu) of recombinant MGF VV. After two weeks, immunized rats are boosted with approximately 10^6 primary rat fibroblasts infected with recombinant MGF VV (greater than 5 pfu per cell). After another two weeks, the rats are boosted with approximately 2×10^6 Ci27 cells that express MGF. Three days later the rats are sacrificed and its spleen cells recovered. The spleen cells are fused with P3X63-Ag8.653 mouse myeloma cells as described

in Examples 10 and 11. Positive hybridoma clones are selected by an assay, such as an ELISA assay.

EXAMPLE 13
ASSAYS FOR DETECTING ANTIBODIES

ELISA Assay

An ELISA assay for screening cells for MGF secretion is conducted by seeding cells into a well plate at a concentration of 4×10^5 cells per well in a 200 µl/well volume. This involves preparing a suspension of 2×10^6 cells/ml in media. Hybridoma supernatants and diluted antisera controls are added to each cell for a 30 minute incubation at room temperature in the wells. Following incubation, the wells are washed and approximately 50 µl/well of antispecies-specific antisera (e.g., Goat anti-rat Peroxidase Bio Rad) are added (diluted 1:1000 in 5% fetal calf serum/PBS). The next incubation step is for approximately 30 minutes at room temperature. After washing, substrate solution (for example O-Phenylenediamine 1 mg/ml Zymed, hydrogen peroxide in citrate buffer or TMB substrate, Kirkegaard and Perry) is added to each well. Positive wells are identified by development of color as determined by absorbance in excess of control wells.

ABC Assay

Wells are coated overnight with 10 µg/ml of goat antispecies-specific IgG (Zymed S San Francisco, CA), and then blocked with 5% non-fat dry milk for one hour. Hybridoma supernatants or diluted monoclonal antibody to MGF is added for one hour. After washing, iodinated MGF is added at approximately 2000 cpm/µl for one hour. After washing, the wells are exposed to film. Positive spots indicate the presence of precipitated MGF protein.

Cross-blocking Assay to Determine Epitopes

The relative epitopes of isolated monoclonal antibodies to MGF are determined by a cross-blocking assay. The assay determines is a particular monoclonal antibody inhibits binding of MGF to other specific antibodies. This assay is conducted by binding approximately 2 ml of specific antibody or hybridoma supernatant preincubated with radiolabeled (iodine) MGF (2000 cpm/ml) to a nitrocellulose sheet (Schleicher & Schuell Keene NH). The sheet is dried and then blocked for one hour in 3% BSA (bovine serum albumin) in PSA (phosphate buffered saline). The sheet is thoroughly washed with PBS and exposed to film. A diminished signal indicates inhibition of one antibody's binding by another.

Dot Blot Immunoassay

A sheet of nitrocellulose membrane (Schleicher & Schuell Keene NH) is marked into squares and approximately 25 ng of MGF is placed onto the membrane within each square. After the MGF is dried, the sheet is blocked with 3% BSA in PBS for one hour. After washing and drying the sheet, specific antibody in the form of hybridoma supernatant or

antibody is placed onto the membrane for 30 minutes. Next, the membrane is washed and excess antibody removed by incubating with PBS. The membrane is next incubated with diluted labeled antisera which is species specific for the particular antibody. For example, if the specific antibody is a mouse monoclonal antibody, the labeled antisera is a 1:2000 of goat anti-mouse antibody conjugated with, for example, a horse radish peroxidase. After incubation, the membrane is washed and color as an indicator is developed with a substrate for the label, such as 4-chloro-1-napthol with an oxidizing agent such as hydrogen peroxide. Positive tests show color and indicate the presence of an antibody specific for MGF coated onto the membrane. This assay is useful for screening of multiple hybridoma colonies.

Radioimmunoprecipitation

This assay determines the presence of an immunoprecipitating antibody. The assay is conducted by adding 50 µl of PBSTA (PBS containing 50 mg/ml BSA and 10 µl/ml Triton X100, 2 µl of rabbit anti-species specific antibody (i.e., rabbit anti-mouse IgG, IgM and IgA, specific antibody (such as 50 µl of hybridoma supernatant, or 2 µl serum, or 2 µl ascites fluid from a mouse innoculated with a hybridoma), 50 µl of 20% Protein A Sepharose solution (Sigma), and 25 µl of radioiodinated MGF (about 2000 cpm/µl). The sample is spun down and then incubated overnight. Following incubation, the sample is washed three times by adding PBS and spinning down the pellet. The pellet is counted with a gamma counter. A positive indicates the presence of an immunoprecipitating antibody.

From the foregoing it will be appreciated that other sequences, embodiments, activities and purification processes can produce polypeptides with MGF biological activities and are included within the scope of the present invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Williams, Douglas E.
Lyman, Stewart

(ii) TITLE OF INVENTION: Mast Cell Growth Factor

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Immunex Corporation
(B) STREET: 51 University Street
(C) CITY: Stattle
(D) STATE: Washington
(E) COUNTRY: USA
(F) ZIP: 98101

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.24

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Oster, Jeffrey B.
(B) REGISTRATION NUMBER: 32585
(C) REFERENCE/DOCKET NUMBER: 0521D

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 2065870430

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1533 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Murine

(vii) IMMEDIATE SOURCE:
(B) CLONE: mMGF-94

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCGCAACGGC CAAGGACGGG GCGCTGCGTT CGAGCTACCC AATGCTGGGA CTATCTGCAG 60

CCGCTGCTGG TGCAATATGC TGGAGCTCCA GAACAGCTAA ACGGAGTCGC CACACCGCTG 120

CCTGGGCTGG ATCGCAGCGC TGCCTTTCCT TATGAAGAAG ACACAAACTT GGATTATCAC 180

TTGCATTTAT TTGTTTCTCT TCAACTGCTC CTATTTAATC CTCTTGTCAA AACCAAGGAG 240

ATCGAATGTG CGGGAATCCT GTGACTGATA ATGTAAAAGA CATTACAAAA CTGCGAATAA 300

AATGTGGCAA ATCTTCCAAA TGACTATATG ATAACCCTCA ACTATGTCAA AAAATTTATA 360

GCCGGGATGG ATGTTTTGCC TAGTCATTGT TGGCTACGAG ATATGAAGTA ACTAGATGTA 420

ATACAATTAT CACTCAGCTT GACTACTCTT CTGGACAAGT TCAGTTATCA AATATTTCTG 480

AAGGCTTGAG TAATTACTCC ATCATAGACA AAAGGATACT TGGGAAAATA GTGGATGACC 540

TCGTGTTATG CATGGAAGAA AACGAAAACT GGAGCACCGA AGAATATAAA AGAATCTCCG 600

AAGAGGCCAG AAACTAGAAA AGAGGTAGTC CTTTACTCCT GAAGAATTCT TTAGTATTTT 660

CAATAGATCC ATTTGGAAGG ATGCCTTTAA GGACTTTATG GTGGCATCTG ACACTAGTGA 720

CTGTAAAATA AAATACGTGC TCTCTTCAAC ATTAGGTCCC GAGAAAGCCA GCTCCCTTAG 780

GATGGAAAGA ATGACAGCAG TAGCAGTAAT AGGAAAGCCG CAAAGGCCCC TGAAAAAAGA 840

AAAAAGGACT CGGGCCTACA ATGGACAGCC ATGGCATTGC CGGCTCTCAT TAGGTTAATA 900

AAATCGCTTG TAATTGGCTT TGCTTTTGGA GCCTTATACT GGAAGAAGAG AAGAATTAAA 960

CAGTCAAGTC TTACAAGGGC AGTTGAAAAT ATACAGATTA ATGTAGAAAG AGAGAAGAGG 1020

ATAATGAGAT AAGTATGTTG CAACAGAAAG AGAGAGAAGG AAGTGGGAGG TTTCAAGAGG 1080

TGTAATTGTG GACGTATCAA CATTGTTACC TTCGCGGAAC AGTGGCTGGT AACAGTTCAT 1140

GTTTGCTTCA TAAATGAAGC AGCCTTAAAC AAATTCCCAT TCTGTCTCAA GTGACAGACC 1200

TCATCCTTAC CTGTTCTTGC TACCCGTGAC CTTGTGTGGA TGATTCAGTT GTTGGAGCAG 1260

AGTGCTTCGC TGTGAACCCT GCACTGAATT ATCATCTGTA AAGAAAAATC TGCACGGAGC 1320

AGGACTCTGG AGGTTTTGCA AGTGATGATA GGGACAAGAA CATGTGTCCA GTCTACTTGC 1380

ACCGTTTGCA TGGCTTGGGA AACGTCTGAG TGCTGAAAAC CCACCCAGCT TTGTTCTTCA 1440

GTCACAACCT GCAGCCTGTC GTTAATTATG GTCTCTGCAA GTAGATTTCA GCCTGGATGG 1500

TGGGGGGAAT TTTTTTTTTC ACAAAAGAGG GAG 1533

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1689 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: N

(vi) ORIGINAL SOURCE:
(A) ORGANISM: MURING MAST CELL GROWTH FACTOR

(vii) IMMEDIATE SOURCE:
(B) CLONE: MMGF-10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CGCAACGGCC AAGGACGGGG CGCTGCGTTC GAGCTACCCA ATGCTGGGAC TATCTGCAGC 60
CGCTGCTGGT GCAATATGCT GGAGCTCCAG AACAGCTAAA CGGAGTCGCC ACACCGCTGC 120
CTGGGCTGGA TCGCAGCGCT GCCTTTCCTT ATGAAGAAGA CACAAACTTG GATTATCACT 180
TGCATTTATT TGTTTCTCTT CAACTGCTCC TATTTAATCC TCTTGTCAAA ACCAAGGAGA 240
TCGAATGTGC GGGAATCCTG TGACTGATAA TGTAAAAGAC ATTACAAAAC TGCGAATAAA 300
ATGTGGCAAA TCTTCCAAAT GACTATATGA TAACCCTCAA CTATGTCAAA AAATTTATAG 360
CCGGGATGGA TGTTTTGCCT AGTCATTGTT GGCTACGAGA TATGAAGTAA CTAGATGTAA 420
TACAATTATC ACTCAGCTTG ACTACTCTTC TGGACAAGTT CAGTTATCAA ATATTTCTGA 480
AGGCTTGAGT AATTACTCCA TCATAGACAA AAGGATACTT GGGAAAATAG TGGATGACCT 540
CGTGTTATGC ATGGAAGAAA ACGAAAACTG GAGCACCGAA GAATATAAAA GAATCTCCGA 600
AGAGGCCAGA AACTAGAAAA GAGGTAGTCC TTTACTCCTG AAGAATTCTT TAGTATTTTC 660
AATAGATCCA TTTGGAAGGA TGCCTTTAAG GACTTTATGG TGGCATCTGA CACTAGTGAC 720
TGTAAAATAA AATACGTGCT CTCTTCAACA TTAGGTCCCG AGAAAGATTC CAGAGTCAGT 780
ATGGAAGAGT CACAAAACCA TTTATGTTAC CCCCTGTTGC AGCCAGCTCC CTTATTAAAA 840
AAGGAATGAC AGCAGTAGCA GTAATAGGAA AGCCGCAAAG GCCCCTAGAA AAGAAAAAAG 900
AAGACTCGGG CCTACAATGG ACAGCCATGG CATTGCCGGC TCTCGAGGTT AATAAAAATT 960
TCGCTTGTAA TTGGCTTTGC TTTTGGAGCC TTATACTGGA AGAGAAGAAT TAAGAAACAG 1020
TCAAGTCTTA CAAGGGCAGT TGAAAATATA CAGATTGTAG AAAGAGAATG AAGAGGATAA 1080
TGAGATAAGT ATGTTGCAAC AGAAAGAGAG AAGGAAGTGG GAGGAATTTC AAGAGGTGTA 1140
ATTGTGGACG TATCAACATT GTTACCTTGG GACGCACAGT GGCTGGTAAC AGTTCATGTT 1200
TGCTTCATAA ATGAAGCAGC CTTAAACAAA TTCCCATTCT GTCTCAAGTG ACAGACCTCA 1260
TCCTTACCTG TTCTTGCTAC CCGTGACCTT GTGTGGATGA TTCAGTTGTT GGAGCAGAGT 1320
GCTTCGCTGT GAACCCTGCA CTGAATTATC ATCTGTAAAG AAAAATCTGC ACGGAGCAGG 1380
ACTCTGGAGG TTTTGCAAGT GATGATAGGG ACAAGAACAT GTGTCCAGTC TACTTGCACC 1440
GTTTGCATGG CTTGGGAAAC GTCTGAGTGC TGAAAACCCA CCCAGCTTTG TTCTTCAGTC 1500
ACAACCTGCA GCCTGTCGTT AATTATGGTC TCTGCAAGTA GATTTCAGCC TGGATGGTGG 1560
GGGGAATTTT TTTTTTCACA AAGGGATGTA GAAAACAATT TAAAAAAACA AAACAAAACA 1620

ATAAAGCGCA ACGGCCAAGG ACGGGGCGCT GCGTTCGAGC TACCCAATGC TGGGACTATC 1680

TGCAGCCGC 1689

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1069 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HUMAN MAST CELL GROWTH FACTOR

(vii) IMMEDIATE SOURCE:
(B) CLONE: HMGF-2D

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ACGGCTGGAT CGCAGCGCTG CCTTTCCTTA TGAAGAAGAC ACAAACTTTG TTGGATTCTC 60

ACTTGCATTT ATCTTCAGCT GCTCCTATTT AATCCTTTCT GACTCGTCAA AACTGAAGGG 120

ATCTGCAGGA ATCGTGTGAC TAATAATATG GCAGAAGATA AGTAAAAGAC GTCACTAAAT 180

TGGTGGCAAA TCTTCCAAAA GACTACAAAT AAAAATATGA TAACCCTCAA ATATGTCCCC 240

GGGATGGATG TTTTGCCAAG TTTTAGTAAC ATTGTTGGAT AAGCGAGATG GTAGTACAAT 300

TGTCAGACAG CTTGCTGTAA GAACTGATCT TCTGGACAAG TTTTCAAATA TTTCTGAAGG 360

CTTGAGTTAA AGGAATTATT CCATCATAGA CAAACTTGTG AATATAGTGG ATGACCTTAT 420

AAAAAAGTGG AGTGCGTGAA AGAAAACTCA TCTAAGGATC TAAAAAAATC AAGCAGAATT 480

CAAGAGCCCA GAACCCAGGC TCTTTACTCC TGAAGAATTC TTTGAGTGGA GAATTTTTAA 540

TAGATCCATT GATGCCTTCA AGGACTTTGT AGTGAGAAGA AAAAAGCATC TGAAACTAGT 600

GATTGTGTGG TTTCTTCAAC ATTAAGTCCT AAGTACAATG AGAAAGGGAA GGCCAAAAAT 660

CCCCCTGGAG ACTCCAGCCT ACACGGAAAG ATGGGCAGCC ATGGCATTGC CAGCATTGTT 720

TTCTCTTATA ATTGGCTAAA ATAAAAGTTT GCTTTTGGAG CCTTATACTG GAAGAAGAGA 780

CAGCCAAGTC TTAAGAATTA GGACAAGGGC AGTTGAAAAT ATACAAATTA ATGAAGAGGA 840

TAATGAGTAG AAAGAGAGGA AGATAAGTAT GTTGCAAGAG AAAGAGAGAG AGTTTCAAGA 900

AGTGTAATGG GAGGGGATTG TGGCTTGTAT CAAGTTTCTC TTCAACTGCT CCTATTTAAT 960

CCTCTTGTCA AAACCAAGGA GATCGAATGC GGCTGGATCG CAGCGCTGCC TTTCCTTATG 1020

AAGAAGACAC AAACTTTGTA TCCAGGAACC ATAGATTCAA CTTTACTTT 1069

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1050 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: N

(vi) ORIGINAL SOURCE:
(A) ORGANISM: HUMAN MAST CELL GROWTH FACTOR

(vii) IMMEDIATE SOURCE:
(B) CLONE: HMGF-2.4

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CGGCTGGATC GCAGCGCTGC CTTTCCTTAT GAAGAAGACA CAAACTTTGT TGGATTCTCA 60

CTTGCATTTA TCTTCAGCTG CTCCTATTTA ATCCTTTCTG ACTCGTCAAA ACTGAAGGGA 120

TCTGCAGGAA TCGTGTGACT AATAATATGG CAGAAGATAA GTAAAAGACG TCACTAAATT 180

GGTGGCAAAT CTTCCAAAAG ACTACAAATA AAAATATGAT AACCCTCAAA TATGTCCCCG 240

GGATGGATGT TTTGCCAAGT TTTAGTAACA TTGTTGGATA AGCGAGATGG TAGTACAATT 300

GTCAGACAGC TTGCTGTAAG AACTGATCTT CTGGACAAGT TTTCAAATAT TTCTGAAGGC 360

TTGAGTTAAA GGAATTATTC CATCATAGAC AAACTTGTGA ATATAGTGGA TGACCTTATA 420

AAAAAGTGGA GTGCGTGAAA GAAAACTCAT CTAAGGATCT AAAAAAATCA AGCAGAATTC 480

AAGAGCCCAG AACCCAGGCT CTTTACTCCT GAAGAATTCT TTGAGTGGAG AATTTTTAAT 540

AGATCCATTG ATGCCTTCAA GGACTTTGTA GTGAGAAGAA AAAAGCATCT GAAACTAGTG 600

ATTGTGTGGT TTCTTCAACA TTAAGTCCTA AGTACAATGA GAAAGATTCC AGAGTCAGTG 660

TCACAAAACC ATTTATGTTA CCCGAAGAAT TCCTGTTGCA GCCAGCTCCC TTAGGAATGA 720

CAGCAGTAGC AGTAATAAAA AAGAAAAGGA AGGCCAAAAA TCCCCCTGGA GACTCCAGCC 780

TACACTGGGC AAGAAAGATA AGCCATGGCA TTGCCAGCAT TGTTTTCTCT TATAATTGGC 840

TTTGCTAATA AAAGAATTTG GAGCCTTATA CTGGAAGAAG AGACAGCCAA GTCTTACAAG 900

GGAATTAGGT AGGCAGTTGA AAATATACAA ATTAATGAAG AGGATAATGA GATAAGTAAA 960

GAGAGGAAGA TGTTGCAAGA GAAAGAGAGA GAGTTTCAAG AAGTGTAATT GTGGTGGGAG 1020

GGGACTTGTA TCAAGACACA AACTTTGTTG 1050

WE CLAIM:

1. An isolated DNA sequence encoding a polypeptide exhibiting human MGF activity, such sequence comprising a DNA sequence selected from the group consisting of:

(a) a DNA sequence encoding a human MGF polypeptide comprising the sequence:

X_n X_n X_n Cys Arg Asn Arg Val Thr Asn Asn
Val Lys Asp Val Thr Lys Leu Val Ala Asn Leu Pro Lys Asp Tyr
Met Ile Thr Leu Lys Tyr Val Pro Gly Met Asp Val Leu Pro Ser
His Cys Trp Ile Ser Glu Met Val Val Gln Leu Ser Asp Ser Leu
Thr Asp Leu Leu Asp Lys Phe Ser Asn Ile Ser Glu Gly Leu Ser
Asn Tyr Ser Ile Ile Asp Lys Leu Val Asn Ile Val Asp Asp Leu
Val R_1 Cys R_2 Q Glu Asn Ser Ser Lys Asp Leu Lys Lys Ser
Phe Lys Ser Pro Glu Pro Arg Leu Phe Thr Pro Glu Glu Phe Phe
Arg Ile Phe Asn Arg Ser Ile Asp Ala Phe Lys Asp Phe Val Val
Ala Ser Glu Thr Ser Asp Cys X_n X_n X_n X_n X_n X_n X_n X_n
X_n X_n

wherein n is an integer 0 or 1, X is a natural amino acid, and R_1 and R_2 are any amino acid, and Q is Lys, Arg or Glu; and

(b) DNA sequences that hybridize to the DNA sequence of (a) under conditions of high stringency, which code on expression for a polypeptide with human MGF activity; and proliferation of human hematopoietic progenitor cells in combination with human interleukin-3 (hIL-3).

2. An isolated DNA sequence according to claim 1, wherein R_1 is Glu, R_2 is Val and Q is Lys.

3. An isolated DNA sequence according to claim 1, wherein the DNA sequence encoding a polypeptide exhibiting human MGF activity comprises a DNA sequence selected from the group consisting of:

(a) the DNA sequence of Sequence I.D. No. 3 from nucleotide 103 to nucleotide 546;

(b) DNA sequences that hybridize under high stringency to the DNA sequence of (a) and which code on expression of a polypeptide with human MGF activity; and

(c) DNA sequences that encode polypeptides encoded by any of the foregoing DNA sequences but vary in nucleotide sequence as a result of degeneracy of the genetic code.

4. An isolated DNA sequence according to claim 3, wherein the DNA sequence encodes human Δ28 MGF.

5. An isolated DNA sequence according to claim 1, wherein the DNA sequence encoding a polypeptide exhibiting human MGF activity comprises a DNA sequence selected from the group consisting of:

(a) the DNA sequence of Sequence I.D. No. 4 from nucleotide 113 to nucleotide 657;

(b) DNA sequences that hybridize under high stringency to the DNA sequence of (a) and which code on expression of a polypeptide with human MGF activity; and

(c) DNA sequences that encode polypeptides encoded by any of the foregoing DNA sequences but vary in nucleotide sequence as a result of degeneracy of the genetic code.

6. An isolated DNA sequence according to claim 5, wherein the DNA sequence encodes a 185 amino acid extracellular domain of hMGF.

7. An isolated DNA sequence encoding MGF or a derivative, analog or allelic variant thereof displaying at least one biological activity selected from the group consisting of:

(a) binding to a receptor protein expressed by a mammalian c-*kit* proto-oncogene;

(b) inducing proliferation of mammalian mast cells;

(c) inducing proliferation of mammalian hematopoietic stem cells; and

(d) inducing proliferation of an IL-3 dependent cell line having the characteristics of MC6.

8. An isolated MGF polypeptide comprising the amino acid sequence:

X_n X_n X_n Cys Arg Asn Arg Val Thr Asn Asn
Val Lys Asp Val Thr Lys Leu Val Ala Asn Leu Pro Lys Asp Tyr
Met Ile Thr Leu Lys Tyr Val Pro Gly Met Asp Val Leu Pro Ser
His Cys Trp Ile Ser Glu Met Val Val Gln Leu Ser Asp Ser Leu
Thr Asp Leu Leu Asp Lys Phe Ser Asn Ile Ser Glu Gly Leu Ser
Asn Tyr Ser Ile Ile Asp Lys Leu Val Asn Ile Val Asp Asp Leu
Val R_1 Cys R_2 Q Glu Asn Ser Ser Lys Asp Leu Lys Lys Ser
Phe Lys Ser Pro Glu Pro Arg Leu Phe Thr Pro Glu Glu Phe Phe
Arg Ile Phe Asn Arg Ser Ile Asp Ala Phe Lys Asp Phe Val Val
Ala Ser Glu Thr Ser Asp Cys X_n X_n X_n X_n X_n X_n X_n X_n
X_n X_n

wherein n is an integer 0 or 1, X is a natural amino acid, R_1 and R_2 are any amino acid, and Q is Lys, Arg or Glu, or an amino acid sequence encoded by an isolated DNA sequence according to claim 1.

9. An isolated MGF polypeptide according to claim 8, wherein R_1 is Glu, R_2 is Val and Q is Lys.

10. An isolated MGF polypeptide according to claim 8 wherein the amino acid sequence is

Glu Gly Ile Cys Arg Asn Arg Val Thr Asn Asn Val Lys Asp Val
Thr Lys Leu Val Ala Asn Leu Pro Lys Asp Tyr Met Ile Thr Leu
Lys Tyr Val Pro Gly Met Asp Val Leu Pro Ser His Cys Trp Ile
Ser Glu Met Val Val Gln Leu Ser Asp Ser Leu Thr Asp Leu Leu
Asp Lys Phe Ser Asn Ile Ser Glu Gly Leu Ser Asn Tyr Ser Ile
Ile Asp Lys Leu Val Asn Ile Val Asp Asp Leu Val Glu Cys Val
Lys Glu Asn Ser Ser Lys Asp Leu Lys Lys Ser Phe Lys Ser Pro
Glu Pro Arg Leu Phe Thr Pro Glu Glu Phe Phe Arg Ile Phe Asn
Arg Ser Ile Asp Ala Phe Lys Asp Phe Val Val Ala Ser Glu Thr
Ser Asp Cys Val Val Ser Ser Thr Leu Ser Pro Glu Lys [Asp Ser
Arg Val Ser Val Thr Lys Pro Phe Met Leu Pro Pro Val Ala Ala
Ser Ser Leu Arg Asn Asp Ser Ser Ser Ser Asn]$_X$ Y Lys Ala Lys
Asn Pro Pro Gly Asp,

wherein x is 0 or 1 and Y is Arg or Gly.

11. An isolated MGF polypeptide according to claim 10, wherein the sequence is Δ28 MGF.

12. A recombinant expression vector comprising an isolated DNA sequence according to claim 1.

13. A host cell comprising a recombinant expression vector according to Claim 12.

14. The host cell of claim 13 wherein the host cell is selected from the group consisting of *E. coli*, *Pseudomonas*, *Bacillus*, *Streptomyces*, yeast, fungi, insect cells and mammalian cells.

15. The host cell of claim 14 wherein the host cell is a yeast cell of the species *Saccharomyces cerevisiae* strains XV2181 or XNN281.

16. A process for purifying recombinant MGF from yeast supernatants comprising:

(a) cation exchange fractionating yeast supernatant containing MGF at an acid pH of from about 2.0-4.0 and eluting with acetate at a pH of 4.5-5.5;

(b) reverse phase high performance liquid chromatography (RP-HPLC) fractionating the anion exchange eluate with a linear gradient of acetonitrile; and

(c) cation exchange fractionating the RP-HPLC eluate in a physiologic pH buffer.

17. A pharmaceutical composition for stimulating proliferation of hematopoietic cells comprising a MGF polypeptide according to claim 8 in combination with a growth factor wherein the growth factor is selected from the group consisting of GM-CSF, G-CSF, IL-3, EPO, IL-6, IL-1α, IL-1β, IL-7, LIF, pIXY-321, and combinations thereof.

18. The pharmaceutical composition of claim 17 wherein the growth factor is IL-3 or pIXY-321.

19. An antibody immunoreactive with MGF according to claim 8.

20. A process for using the DNA sequence of claim 1 comprising expressing the DNA sequence of claim 1 in an expression vector and a host cell and recovering recombinant polypeptide exhibiting human MGF activity.

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Figure 1

```
                                                    GC AAC GGC   8

CAA GGA CGG GGC GCT GCG TTC GAG CTA CCC AAT GCT GGG ACT ATC 53

TGC AGC CGC TGC TGG TGC AAT ATG CTG GAG CTC CAG AAC AGC TAA 98

ACG GAG TCG CCA CAC CGC TGC CTG GGC TGG ATC GCA GCG CTG CCT 143

TTC CTT ATG AAG AAG ACA CAA ACT TGG ATT ATC ACT TGC ATT TAT 188
      Met Lys Lys Thr Gln Thr Trp Ile Ile Thr Cys Ile Tyr -13

CTT CAA CTG CTC CTA TTT AAT CCT CTT GTC AAA ACC AAG GAG ATC 233
Leu Gln Leu Leu Leu Phe Asn Pro Leu Val Lys Thr Lys Glu Ile   3

TGC GGG AAT CCT GTG ACT GAT AAT GTA AAA GAC ATT ACA AAA CTG 278
Cys Gly Asn Pro Val Thr Asp Asn Val Lys Asp Ile Thr Lys Leu  18

GTG GCA AAT CTT CCA AAT GAC TAT ATG ATA ACC CTC AAC TAT GTC 323
Val Ala Asn Leu Pro Asn Asp Tyr Met Ile Thr Leu Asn Tyr Val  33

GCC GGG ATG GAT GTT TTG CCT AGT CAT TGT TGG CTA CGA GAT ATG 368
Ala Gly Met Asp Val Leu Pro Ser His Cys Trp Leu Arg Asp Met  48

GTA ATA CAA TTA TCA CTC AGC TTG ACT ACT CTT CTG GAC AAG TTC 413
Val Ile Gln Leu Ser Leu Ser Leu Thr Thr Leu Leu Asp Lys Phe  63

TCA AAT ATT TCT GAA GGC TTG AGT AAT TAC TCC ATC ATA GAC AAA 458
Ser Asn Ile Ser Glu Gly Leu Ser Asn Tyr Ser Ile Ile Asp Lys  78

CTT GGG AAA ATA GTG GAT GAC CTC GTG TTA TGC ATG GAA GAA AAC 503
Leu Gly Lys Ile Val Asp Asp Leu Val Leu Cys Met Glu Glu Asn  93

GCA CCG AAG AAT ATA AAA GAA TCT CCG AAG AGG CCA GAA ACT AGA 548
Ala Pro Lys Asn Ile Lys Glu Ser Pro Lys Arg Pro Glu Thr Arg 108

TCC TTT ACT CCT GAA GAA TTC TTT AGT ATT TTC AAT AGA TCC ATT 593
Ser Phe Thr Pro Glu Glu Phe Phe Ser Ile Phe Asn Arg Ser Ile 123

GAT GCC TTT AAG GAC TTT ATG GTG GCA TCT GAC ACT AGT GAC TGT 638
Asp Ala Phe Lys Asp Phe Met Val Ala Ser Asp Thr Ser Asp Cys 138

GTG CTC TCT TCA ACA TTA GGT CCC GAG AAA GCC AGC TCC CTT AGG 683
Val Leu Ser Ser Thr Leu Gly Pro Glu Lys Ala Ser Ser Leu Arg 153

AAT GAC AGC AGT AGC AGT AAT AGG AAA GCC GCA AAG GCC CCT GAA 728
Asn Asp Ser Ser Ser Ser Asn Arg Lys Ala Ala Lys Ala Pro Glu 168

GAC TCG GGC CTA CAA TGG ACA GCC ATG GCA TTG CCG GCT CTC ATT 773
Asp Ser Gly Leu Gln Trp Thr Ala Met Ala Leu Pro Ala Leu Ile 183
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Figure 1 (continued)

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TCG CTT GTA ATT GGC TTT GCT TTT GGA GCC TTA TAC TGG AAG AAG 818
Ser Leu Val Ile Gly Phe Ala Phe Gly Ala Leu Tyr Trp Lys Lys 198

AAA CAG TCA AGT CTT ACA AGG GCA GTT GAA AAT ATA CAG ATT AAT 863
Lys Gln Ser Ser Leu Thr Arg Ala Val Glu Asn Ile Gln Ile Asn 213

GAA GAG GAT AAT GAG ATA AGT ATG TTG CAA CAG AAA GAG AGA GAA 908
Glu Glu Asp Asn Glu Ile Ser Met Leu Gln Gln Lys Glu Arg Glu 228

TTT CAA GAG GTG TAA TTG TGG ACG TAT CAA CAT TGT TAC CTT CGC 953
Phe Gln Glu Val End                                         232

ACA GTG GCT GGT AAC AGT TCA TGT TTG CTT CAT AAA TGA AGC AGC 998

CTT AAA CAA ATT CCC ATT CTG TCT CAA GTG ACA GAC CTC ATC CTT 1043

ACC TGT TCT TGC TAC CCG TGA CCT TGT GTG GAT GAT TCA GTT GTT 1088

GGA GCA GAG TGC TTC GCT GTG AAC CCT GCA CTG AAT TAT CAT CTG 1133

TAA AGA AAA ATC TGC ACG GAG CAG GAC TCT GGA GGT TTT GCA AGT 1178

GAT GAT AGG GAC AAG AAC ATG TGT CCA GTC TAC TTG CAC CGT TTG 1223

CAT GGC TTG GGA AAC GTC TGA GTG CTG AAA ACC CAC CCA GCT TTG 1268

TTC TTC AGT CAC AAC CTG CAG CCT GTC GTT AAT TAT GGT CTC TGC 1313

AAG TAG ATT TCA GCC TGG ATG GTG GGG GGA ATT TTT TTT TTC ACA 1358

AA                                                          1360
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Figure 2

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                                                GC AAC GGC      8

CAA GGA CGG GGC GCT GCG TTC GAG CTA CCC AAT GCT GGG ACT ATC  53

TGC AGC CGC TGC TGG TGC AAT ATG CTG GAG CTC CAG AAC AGC TAA  98

ACG GAG TCG CCA CAC CGC TGC CTG GGC TGG ATC GCA GCG CTG CCT 143

TTC CTT ATG AAG AAG ACA CAA ACT TGG ATT ATC ACT TGC ATT TAT 188
       Met Lys Lys Thr Gln Thr Trp Ile Ile Thr Cys Ile Tyr -13

CTT CAA CTG CTC CTA TTT AAT CCT CTT GTC AAA ACC AAG GAG ATC 233
Leu Gln Leu Leu Leu Phe Asn Pro Leu Val Lys Thr Lys Glu Ile   3

TGC GGG AAT CCT GTG ACT GAT AAT GTA AAA GAC ATT ACA AAA CTG 278
Cys Gly Asn Pro Val Thr Asp Asn Val Lys Asp Ile Thr Lys Leu  18

GTG GCA AAT CTT CCA AAT GAC TAT ATG ATA ACC CTC AAC TAT GTC 323
Val Ala Asn Leu Pro Asn Asp Tyr Met Ile Thr Leu Asn Tyr Val  33

GCC GGG ATG GAT GTT TTG CCT AGT CAT TGT TGG CTA CGA GAT ATG 368
Ala Gly Met Asp Val Leu Pro Ser His Cys Trp Leu Arg Asp Met  48

GTA ATA CAA TTA TCA CTC AGC TTG ACT ACT CTT CTG GAC AAG TTC 413
Val Ile Gln Leu Ser Leu Ser Leu Thr Thr Leu Leu Asp Lys Phe  63

TCA AAT ATT TCT GAA GGC TTG AGT AAT TAC TCC ATC ATA GAC AAA 458
Ser Asn Ile Ser Glu Gly Leu Ser Asn Tyr Ser Ile Ile Asp Lys  78

CTT GGG AAA ATA GTG GAT GAC CTC GTG TTA TGC ATG GAA GAA AAC 503
Leu Gly Lys Ile Val Asp Asp Leu Val Leu Cys Met Glu Glu Asn  93

GCA CCG AAG AAT ATA AAA GAA TCT CCG AAG AGG CCA GAA ACT AGA 548
Ala Pro Lys Asn Ile Lys Glu Ser Pro Lys Arg Pro Glu Thr Arg 108

TCC TTT ACT CCT GAA GAA TTC TTT AGT ATT TTC AAT AGA TCC ATT 593
Ser Phe Thr Pro Glu Glu Phe Phe Ser Ile Phe Asn Arg Ser Ile 123

GAT GCC TTT AAG GAC TTT ATG GTG GCA TCT GAC ACT AGT GAC TGT 638
Asp Ala Phe Lys Asp Phe Met Val Ala Ser Asp Thr Ser Asp Cys 138

GTG CTC TCT TCA ACA TTA GGT CCC GAG AAA GAT TCC AGA GTC AGT 683
Val Leu Ser Ser Thr Leu Gly Pro Glu Lys Asp Ser Arg Val Ser 153

GTC ACA AAA CCA TTT ATG TTA CCC CCT GTT GCA GCC AGC TCC CTT 728
Val Thr Lys Pro Phe Met Leu Pro Pro Val Ala Ala Ser Ser Leu 168

AGG AAT GAC AGC AGT AGC AGT AAT AGG AAA GCC GCA AAG GCC CCT 773
Arg Asn Asp Ser Ser Ser Ser Asn Arg Lys Ala Ala Lys Ala Pro 183
```

Figure 2 (continued)

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GAA GAC TCG GGC CTA CAA TGG ACA GCC ATG GCA TTG CCG GCT CTC 818
Glu Asp Ser Gly Leu Gln Trp Thr Ala Met Ala Leu Pro Ala Leu 198

ATT TCG CTT GTA ATT GGC TTT GCT TTT GGA GCC TTA TAC TGG AAG 863
Ile Ser Leu Val Ile Gly Phe Ala Phe Gly Ala Leu Tyr Trp Lys 213

AAG AAA CAG TCA AGT CTT ACA AGG GCA GTT GAA AAT ATA CAG ATT 908
Lys Lys Gln Ser Ser Leu Thr Arg Ala Val Glu Asn Ile Gln Ile 228

AAT GAA GAG GAT AAT GAG ATA AGT ATG TTG CAA CAG AAA GAG AGA 953
Asn Glu Glu Asp Asn Glu Ile Ser Met Leu Gln Gln Lys Glu Arg 243

GAA TTT CAA GAG GTG TAA TTG TGG ACG TAT CAA CAT TGT TAC CTT 998
Glu Phe Gln Glu Val End                                     248

CGC ACA GTG GCT GGT AAC AGT TCA TGT TTG CTT CAT AAA TGA AGC 1043

AGC CTT AAA CAA ATT CCC ATT CTG TCT CAA GTG ACA GAC CTC ATC 1088

CTT ACC TGT TCT TGC TAC CCG TGA CCT TGT GTG GAT GAT TCA GTT 1133

GTT GGA GCA GAG TGC TTC GCT GTG AAC CCT GCA CTG AAT TAT CAT 1178

CTG TAA AGA AAA ATC TGC ACG GAG CAG GAC TCT GGA GGT TTT GCA 1223

AGT GAT GAT AGG GAC AAG AAC ATG TGT CCA GTC TAC TTG CAC CGT 1268

TTG CAT GGC TTG GGA AAC GTC TGA GTG CTG AAA ACC CAC CCA GCT 1313

TTG TTC TTC AGT CAC AAC CTG CAG CCT GTC GTT AAT TAT GGT CTC 1358

TGC AAG TAG ATT TCA GCC TGG ATG GTG GGG GGA ATT TTT TTT TTC 1403

ACA AAG GGA TGT AGA AAA CAA TTT AAA AAA ACA AAA CAA AAC AAT 1448

AA                                                          1450
```

Figure 3

```
GGC TGG ATC GCA GCG CTG CCT TTC CTT ATG AAG AAG ACA CAA ACT   45
                                Met Lys Lys Thr Gln Thr  -20

TGG ATT CTC ACT TGC ATT TAT CTT CAG CTG CTC CTA TTT AAT CCT   90
Trp Ile Leu Thr Cys Ile Tyr Leu Gln Leu Leu Leu Phe Asn Pro   -5

CTC GTC AAA ACT GAA GGG ATC TGC AGG AAT CGT GTG ACT AAT AAT  135
Leu Val Lys Thr Glu Gly Ile Cys Arg Asn Arg Val Thr Asn Asn   11

GTA AAA GAC GTC ACT AAA TTG GTG GCA AAT CTT CCA AAA GAC TAC  180
Val Lys Asp Val Thr Lys Leu Val Ala Asn Leu Pro Lys Asp Tyr   26

ATG ATA ACC CTC AAA TAT GTC CCC GGG ATG GAT GTT TTG CCA AGT  225
Met Ile Thr Leu Lys Tyr Val Pro Gly Met Asp Val Leu Pro Ser   41

CAT TGT TGG ATA AGC GAG ATG GTA GTA CAA TTG TCA GAC AGC TTG  270
His Cys Trp Ile Ser Glu Met Val Val Gln Leu Ser Asp Ser Leu   56

ACT GAT CTT CTG GAC AAG TTT TCA AAT ATT TCT GAA GGC TTG AGT  315
Thr Asp Leu Leu Asp Lys Phe Ser Asn Ile Ser Glu Gly Leu Ser   71

AAT TAT TCC ATC ATA GAC AAA CTT GTG AAT ATA GTG GAT GAC CTT  360
Asn Tyr Ser Ile Ile Asp Lys Leu Val Asn Ile Val Asp Asp Leu   86

GTG GAG TGC GTG AAA GAA AAC TCA TCT AAG GAT CTA AAA AAA TCA  405
Val Glu Cys Val Lys Glu Asn Ser Ser Lys Asp Leu Lys Lys Ser  101

TTC AAG AGC CCA GAA CCC AGG CTC TTT ACT CCT GAA GAA TTC TTT  450
Phe Lys Ser Pro Glu Pro Arg Leu Phe Thr Pro Glu Glu Phe Phe  116

AGA ATT TTT AAT AGA TCC ATT GAT GCC TTC AAG GAC TTT GTA GTG  495
Arg Ile Phe Asn Arg Ser Ile Asp Ala Phe Lys Asp Phe Val Val  131

GCA TCT GAA ACT AGT GAT TGT GTG GTT TCT TCA ACA TTA AGT CCT  540
Ala Ser Glu Thr Ser Asp Cys Val Val Ser Ser Thr Leu Ser Pro  146

GAG AAA GGG AAG GCC AAA AAT CCC CCT GGA GAC TCC AGC CTA CAC  585
Glu Lys Gly Lys Ala Lys Asn Pro Pro Gly Asp Ser Ser Leu His  161

TGG GCA GCC ATG GCA TTG CCA GCA TTG TTT TCT CTT ATA ATT GGC  630
Trp Ala Ala Met Ala Leu Pro Ala Leu Phe Ser Leu Ile Ile Gly  176

TTT GCT TTT GGA GCC TTA TAC TGG AAG AAG AGA CAG CCA AGT CTT  675
Phe Ala Phe Gly Ala Leu Tyr Trp Lys Lys Arg Gln Pro Ser Leu  191

ACA AGG GCA GTT GAA AAT ATA CAA ATT AAT GAA GAG GAT AAT GAG  720
Thr Arg Ala Val Glu Asn Ile Gln Ile Asn Glu Glu Asp Asn Glu  206
```

Figure 3 (continued)

```
ATA AGT ATG TTG CAA GAG AAA GAG AGA GAG TTT CAA GAA GTG TAA  765
Ile Ser Met Leu Gln Glu Lys Glu Arg Glu Phe Gln Glu Val      220

TTG TGG CTT GTA TCA                                        780
```

Figure 4

```
GGC TGG ATC GCA GCG CTG CCT TTC CTT ATG AAG AAG ACA CAA ACT   45
                                Met Lys Lys Thr Gln Thr  -20

TGG ATT CTC ACT TGC ATT TAT CTT CAG CTG CTC CTA TTT AAT CCT   90
Trp Ile Leu Thr Cys Ile Tyr Leu Gln Leu Leu Leu Phe Asn Pro   -5

CTC GTC AAA ACT GAA GGG ATC TGC AGG AAT CGT GTG ACT AAT AAT  135
Leu Val Lys Thr Glu Gly Ile Cys Arg Asn Arg Val Thr Asn Asn   11

GTA AAA GAC GTC ACT AAA TTG GTG GCA AAT CTT CCA AAA GAC TAC  180
Val Lys Asp Val Thr Lys Leu Val Ala Asn Leu Pro Lys Asp Tyr   26

ATG ATA ACC CTC AAA TAT GTC CCC GGG ATG GAT GTT TTG CCA AGT  225
Met Ile Thr Leu Lys Tyr Val Pro Gly Met Asp Val Leu Pro Ser   41

CAT TGT TGG ATA AGC GAG ATG GTA GTA CAA TTG TCA GAC AGC TTG  270
His Cys Trp Ile Ser Glu Met Val Val Gln Leu Ser Asp Ser Leu   56

ACT GAT CTT CTG GAC AAG TTT TCA AAT ATT TCT GAA GGC TTG AGT  315
Thr Asp Leu Leu Asp Lys Phe Ser Asn Ile Ser Glu Gly Leu Ser   71

AAT TAT TCC ATC ATA GAC AAA CTT GTG AAT ATA GTG GAT GAC CTT  360
Asn Tyr Ser Ile Ile Asp Lys Leu Val Asn Ile Val Asp Asp Leu   86

GTG GAG TGC GTG AAA GAA AAC TCA TCT AAG GAT CTA AAA AAA TCA  405
Val Glu Cys Val Lys Glu Asn Ser Ser Lys Asp Leu Lys Lys Ser  101

TTC AAG AGC CCA GAA CCC AGG CTC TTT ACT CCT GAA GAA TTC TTT  450
Phe Lys Ser Pro Glu Pro Arg Leu Phe Thr Pro Glu Glu Phe Phe  116

AGA ATT TTT AAT AGA TCC ATT GAT GCC TTC AAG GAC TTT GTA GTG  495
Arg Ile Phe Asn Arg Ser Ile Asp Ala Phe Lys Asp Phe Val Val  131

GCA TCT GAA ACT AGT GAT TGT GTG GTT TCT TCA ACA TTA AGT CCT  540
Ala Ser Glu Thr Ser Asp Cys Val Val Ser Ser Thr Leu Ser Pro  146

GAG AAA GAT TCC AGA GTC AGT GTC ACA AAA CCA TTT ATG TTA CCC  585
Glu Lys Asp Ser Arg Val Ser Val Thr Lys Pro Phe Met Leu Pro  161

CCT GTT GCA GCC AGC TCC CTT AGG AAT GAC AGC AGT AGC AGT AAT  630
Pro Val Ala Ala Ser Ser Leu Arg Asn Asp Ser Ser Ser Ser Asn  176

AGG AAG GCC AAA AAT CCC CCT GGA GAC TCC AGC CTA CAC TGG GCA  675
Arg Lys Ala Lys Asn Pro Pro Gly Asp Ser Ser Leu His Trp Ala  191

GCC ATG GCA TTG CCA GCA TTG TTT TCT CTT ATA ATT GGC TTT GCT  720
Ala Met Ala Leu Pro Ala Leu Phe Ser Leu Ile Ile Gly Phe Ala  206
```

Figure 4 (continued)

```
TTT GGA GCC TTA TAC TGG AAG AAG AGA CAG CCA AGT CTT ACA AGG  765
Phe Gly Ala Leu Tyr Trp Lys Lys Arg Gln Pro Ser Leu Thr Arg  221

GCA GTT GAA AAT ATA CAA ATT AAT GAA GAG GAT AAT GAG ATA AGT  810
Ala Val Glu Asn Ile Gln Ile Asn Glu Glu Asp Asn Glu Ile Ser  236

ATG TTG CAA GAG AAA GAG AGA GAG TTT CAA GAA GTG TAA TTG TGG  855
Met Leu Gln Glu Lys Glu Arg Glu Phe Gln Glu Val              248

CTT GTA TCA                                                 864
```

Figure 5

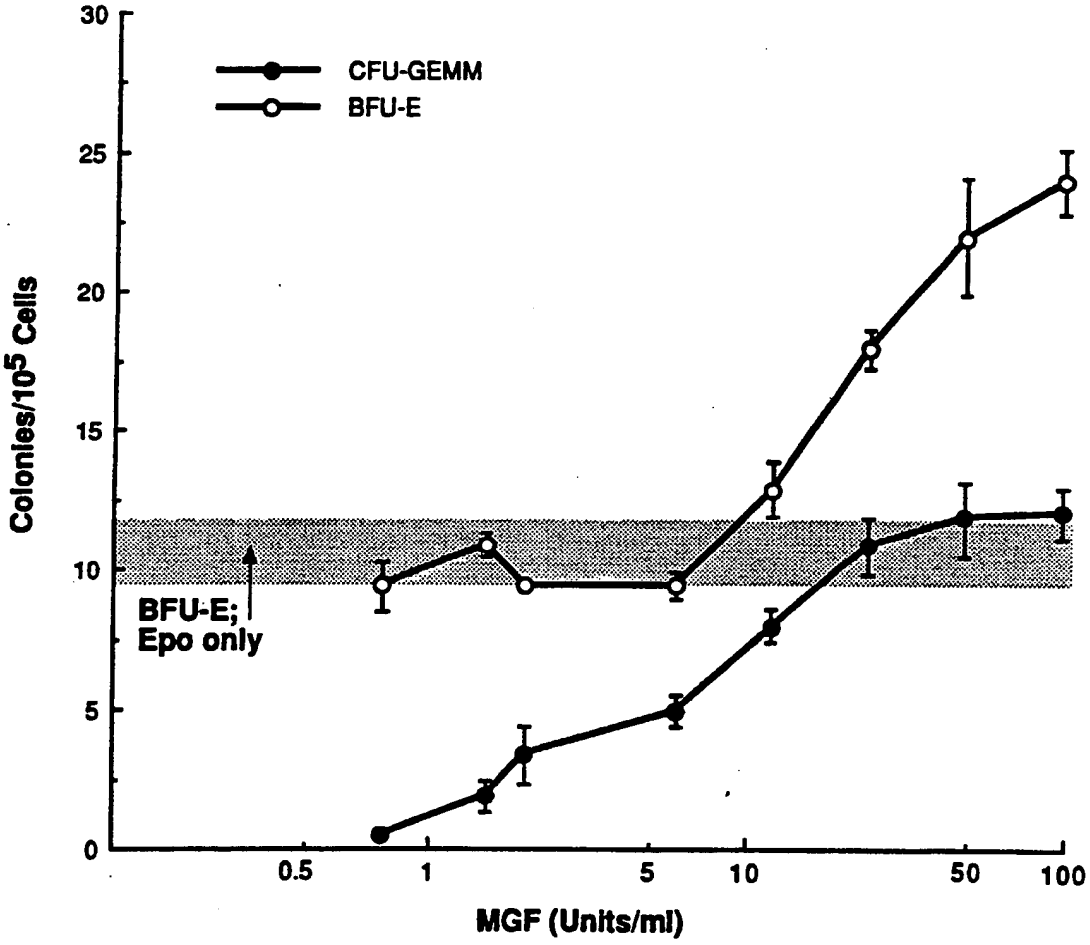
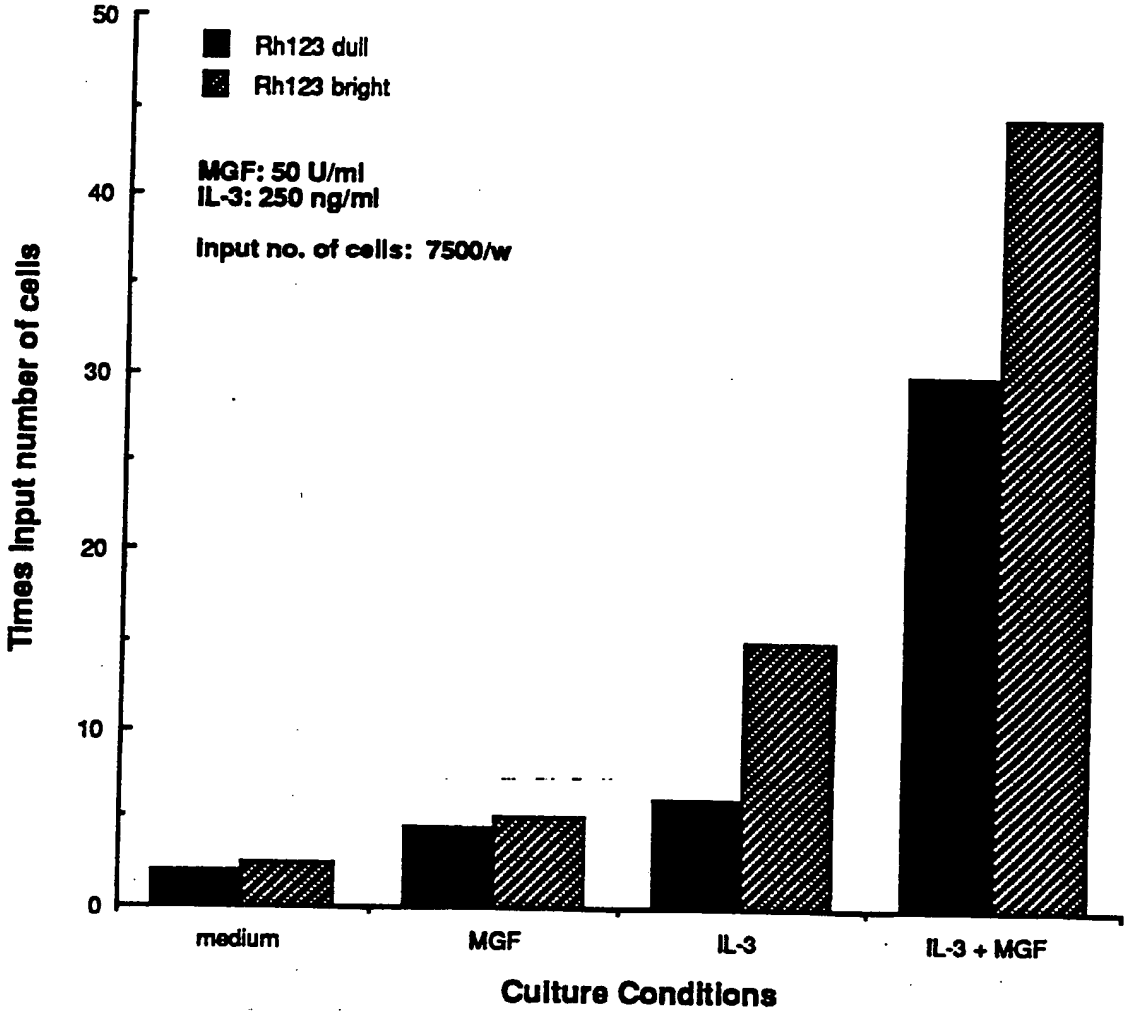

CFU-GEMM
BFU-E
Colonies/10^5 Cells
30
25
20
15
10
5
0
BFU-E;
Epo only
0.5
1
5
10
50
100
MGF (Units/ml)

Figure 6A

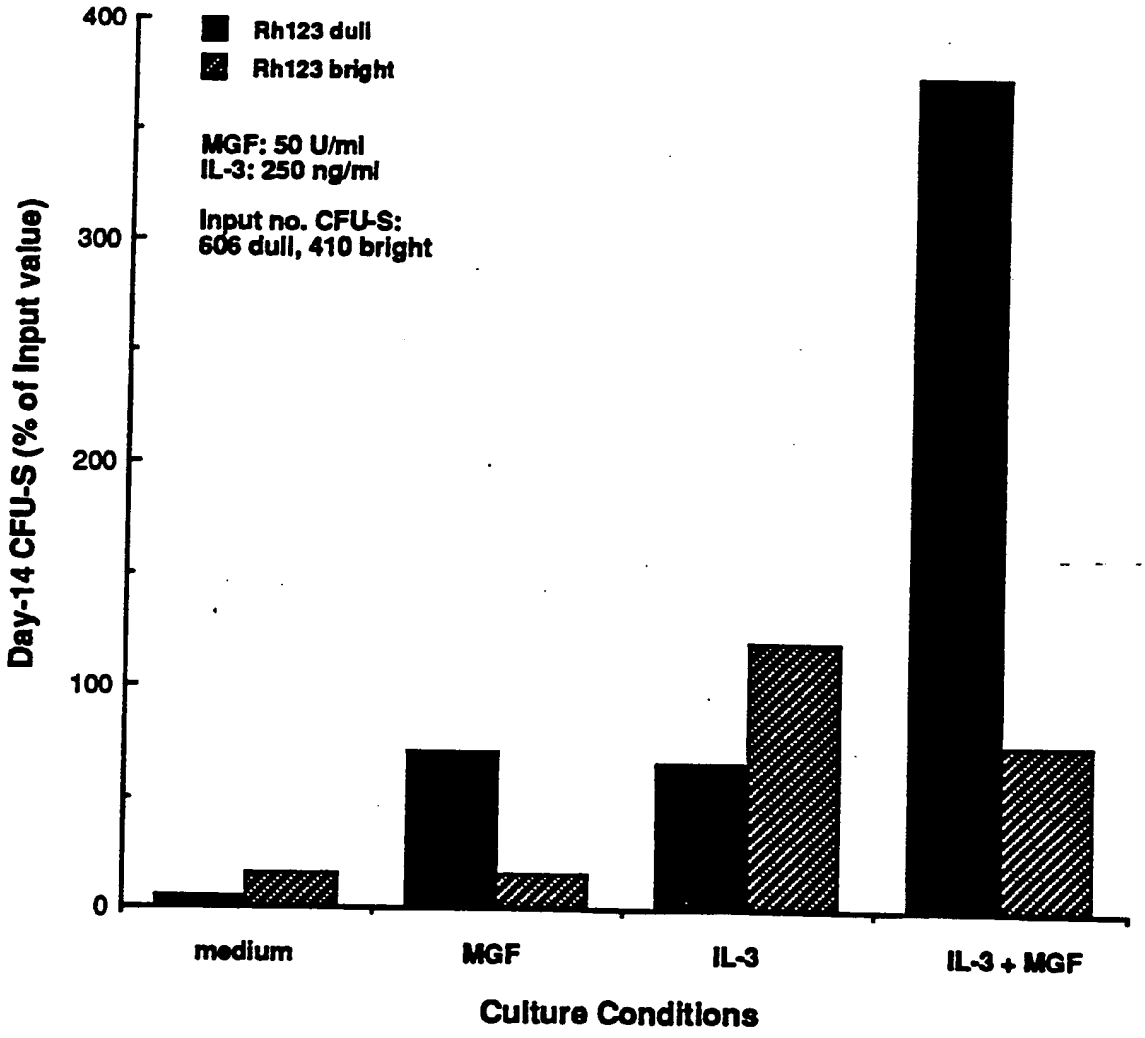
Production of nucleated cells in purified stem cell fractions after 3 days in liquid culture



11/11

Figure 6B

Day-14 CFU-S in purified stem cell fractions after 3 days in liquid culture



INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/04274

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/12 ; C12P21/02 ; A61K37/24 ; C12P21/08

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C12P ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
P,X	EP,A,423 980 (AMGEN INC.) see the whole document, especially figures 42 and 44 ---	1-20
P,X	CELL. vol. 63, October 5, 1990, CAMBRIDGE, NA US pages 235 - 243; ANDERSON, D.M. ET AL.: 'Molecular cloning of mast cell growth factor, a hematopoietin that is active in both membrane bound and soluble forms' see the whole document --- -/--	1,7-8, 12-15,20

* Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
02 OCTOBER 1991	11 OCT 1991
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	ANDRES S.M.

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	CELL. vol. 63, October 5, 1990, CAMBRIDGE, NA US pages 225 - 233; HUANG, E. ET AL.: 'The hematopoietic growth factor KL is encoded by the SI locus and is the ligand of the c-kit receptor, the gene product of the W locus ' see the whole document	1,3,7-8, 12-14,20
P,X	CELL. vol. 63, October 5, 1990, CAMBRIDGE, NA US pages 203 - 211; MARTIN, F. H. ET AL.: 'Primary structure and functional expression of rat and human stem cell factor DNAs ' see the whole document	1-3, 5-10, 12-14, 17-18,20
P,X	CELL. vol. 63, October 5, 1990, CAMBRIDGE, NA US pages 195 - 201; ZSEBO, K. M. ET AL.: 'Identification, purification, and biological characterization of hematopoietic stem cell factor from Buffalo rat liver- conditioned medium ' see the whole document	1,3-4, 7-8, 17-18
P,A		16

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9104274
SA 49517

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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02/10/91

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-423980	24-04-91	AU-A- 6541090	16-05-91
		WO-A- 9105795	02-05-91